REMARKS

Explanation of Amendments

The application was subject to a restriction requirement that identified six groups: Groups 1 and 2 were drawn to compounds wherein X is N or CH, respectively; Groups III and IV were drawn to complex compositions comprising compounds of Groups I and II, respectively; and Groups V and VI were drawn to the additional uses of claim 18 employing compounds of Groups I and II, respectively.

Applicants affirm the election of Group V, and request the entry of the above amendments in order to comply with the restriction requirement. Applicants recognize that claims 1-17, 19 and 20 were withdrawn since, as filed, they fell into non-elected groups. Applicants urge, however, that when amended as requested to read as method of treatment claims and when the scope is amended in view of the restriction requirement regarding X, they should be considered as a part of Group V.

The specification is amended to update the status of the parent application. The requested amendment should overcome the objection to the disclosure.

Claims 14 and 16 are canceled in view of their allowance in the parent application.

Claims 15 and 17-20 are canceled as being drawn to non-elected inventions, i.e., combinations of actives.

Claims 1 and 18 are canceled and replaced with new independent claim 21 which incorporates the definition of the compounds of formula I from claim 1 in the method of claim 18. This amendment overcomes the rejection of claim 18 for being dependent on a non-elected claim.

The scope of formula I in claim 21 is amended compared to original claim 1 in view of the restriction to compounds of Group I, and to correspond to the scope of compounds claimed in the granted parent, US 6,720,325. That is, claim 21 includes "X is nitrogen" in the line before the definition of Q and Z, and deletes ", X" from original line 6. Limiting "X" to nitrogen necessitated the deletion of =CH(C_1-C_6)alkyl and the subsequent proviso from the definition of R^2 and R^3 together, as well as requiring the deletion of the additional R^3 definitions applicable when X and Z are both CH. Deleting the additional definitions of R^3 necessitated the deletion of the variable R^{13}.

Also in claim 21, the term "isomers" was replaced with the terms "diastereomer," "enantiomer," and "atropisomer" as defined on page 11, lines 18-21 of the specification.

The definition of R^4 was amended to be $(C_1\text{-}C_6)$alkyl in view of the preferences cited on page 5, line 17, of the specification. In view of that amendment, the reference to R^4 was deleted from the original combined definition of R and R^4-R^7.

The definition of R^8 in claim 21 includes only the preferred definitions identified on page 5, lines 23-14, and a definition of heteroaryl based on the preferences recited on page 5, lines 19-21 was added at the end of the claim (i.e., heteroaryl is defined in claim 21 as thienyl, pyridyl or pyrimidyl).

Claims 2-13 are each amended to be method claims, and claims 2-9, 12 and 13 are amended to be dependent on new claim 21. Claim 13 is also amended to replace the "R^6" variables in the structures in the claims as filed with "CH_3." The amended structures correspond to Examples 1BF and 1BN, and are included in claim 12 as filed (page 40, 9th compound from the bottom and the last compound)

New claim 21 claims the use of compounds of formula I to treat solid organ transplant rejection, arthritis, rheumatoid arthritis or multiple sclerosis, as discussed further below.

Traversal of Rejection:

Claim 18 was rejected under 35 U.S.C. 112, first paragraph, for lack of enablement due to the breadth of the scope and the use of the terms "isomers" and "heteroaryl." In view of the above-requested amendments, especially since the parent application claiming the same scope of compounds has been granted, applicants urge that the compounds claimed are enabled.

Claim 18 was also rejected under 35 U.S.C. 112 for lack of enablement for the uses claimed. Applicants have amended the claims to include only indications where consistent positive statements about a given utility are found in the art, i.e. transplant rejection, arthritis, rheumatoid arthritis (RA) and multiple sclerosis (MS). Applicants submit copies of several references in support of the use of CCR5 antagonists for treating arthritis and MS; since the examiner appears to have accepted treatment of organ transplantation rejection, no reference for that indication is included. The references and brief explanations are as follows (a copy of each reference is provided):

Arthritis, rheumatoid arthritis:

Bruhl et al, Journal of Immunology, 166 (2001) 2420-2426 (reports that depletion of CCR5 cells could treat chronic inflammatory diseases, such as RA)

Also see the references cited on page 34 of the specification, lines 25-27; copies were provided with the IDS.

Multiple sclerosis:

Bruhl et al, Journal of Immunology, 166 (2001) 2420-2426 (reports that depletion of CCR5 cells could treat chronic inflammatory diseases such as MS

Balashov et al, Proc. Natl. Acad. Sci., USA, 96 (1999) 6873-6878 (reports that CCR5 cells are increased in MS, suggesting a role for CCR5 antagonists in treating MS)

Sorensen et al, Journal of Clinical Investigation, 103 (1999), 807-815 (reports that CCR5 cells are increased in MS, suggesting a role for CCR5 antagonists in treating MS)

Simpson et al, Journal of Neuroimmunology, 108 (2000) 192-200 (reports on the elevation of CCR5 cells in MS)

Sellebjerg et al, Journal of Neuroimmunology, 102 (2000) 98-106 (reports that while having the CCR5Δ32 allele does not prevent MS, it does lower the risk, suggesting a role for CCR5 antagonists in treating MS)

Barcellos et al, Immunogenetics, 51 (2000) 281-288 (reports that while having the CCR5 Δ32 allele does not prevent MS, it may delay onset, suggesting a role for CCR5 antagonists in treating MS).

Zang et al, Brain, 123 (2000) 1874-1882 (results suggest that overexpression of CCR5 receptors is critical to aberrant migration of MS-derived T cells to inflammation site)

Bennetts et al, Human Immunology, 58 (1997) 52-59 (reports that the CCR5Δ32 allele does not protect against MS; this statement is addressed by the later reports, above, that although it does not prevent MS, having the allele lowers the risk and/or may treat the disease).

It is noted that some references strongly support the indication and other references are less certain about the conclusions. Applicants point out that scientific journal

articles typically use cautious language in describing the implications of their findings, even when the test results are positive.

Applicants also enclose a copy of a manuscript titled "A small molecular weight antagonist of CCR5 inhibits the development of CIA in rhesus monkeys" authored by employees of the assignee and others; it has been submitted for publication, but not yet accepted. The particular CCR5 antagonist discussed in the paper is not claimed in the present invention, but the paper is offered as further proof that CCR5 antagonists are useful in treating arthritis, particularly RA. It is reported that in the CIA (collagen-induced arthritis) model in the rhesus monkey, an accepted model for RA, the CCR5 antagonist tested reduced the incidence and severity of CIA. Those skilled in the art would not find it unreasonable or unbelievable that other compounds demonstrated to be CCR5 antagonists would also be useful in treating arthritis.

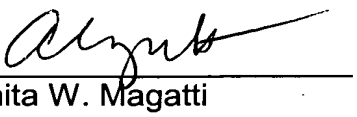
Applicants urge that the references cited are, at the least, predictive of the use of CCR5 antagonists in the treatment of arthritis, RA and MS. The rejection points to the statement in the Wang et al reference saying that "the relative contribution made by individual chemokine receptors to the progression of synovitis is not fully known," but this does not mean that CCR5 antagonists do *not* make a contribution to treating it. Also, the abstract reports that higher expression levels of CCR5 in synovial fluids were consistently present in all RA patients, which would lead one skilled in the art to expect that suppressing CCR5 levels could treat RA.

With respect to treatment of MS, the fact that the publication cited in the rejection states that no "robust gene linkages" have been identified does not mean that CCR5 receptors do not contribute in some part to MS. Despite that fact that researchers could not identify a clear link between the CCR5 mutation and MS, the publication states that the CCR5 chemokine is expressed in MS patients with a particular type of lesion; if it is determined after further study that a CCR5 antagonists could only be useful in that type of MS, it would still be useful.

Applicants urge that positive results indicate that the claimed utilities are not unbelievable or unreasonable, and that in the absence of negative results for the claimed utilities, applicants should be entitled to the scope of utilities claimed.

Reconsideration and withdrawal of the rejection under 35 U.S.C. 112, first paragraph, are respectfully requested.

Respectfully submitted,



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Depletion of CCR5-Expressing Cells with Bispecific Antibodies and Chemokine Toxins: A New Strategy in the Treatment of Chronic Inflammatory Diseases and HIV

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The chemokine receptor CCR5 is expressed on the majority of T cells and monocytes in the inflammatory infiltrate of diseases such as rheumatoid arthritis, renal diseases, and multiple sclerosis. In contrast, little expression of CCR5 is found on peripheral blood leukocytes. A specific depletion of CCR5$^+$ cells could therefore be a useful strategy to reduce the cellular infiltrate in chronic inflammations. Moreover, CCR5 is the major coreceptor for M-tropic HIV-1 strains. Depletion of CCR5$^+$ leukocytes may help to eliminate cells latently infected with HIV-1. We designed two constructs that specifically destroy chemokine receptor-positive cells. The first construct, a bispecific Ab, binds simultaneously to CCR5 and CD3. Thereby it redirects CD3$^+$ T cells against CCR5$^+$ target cells. The Ab specifically depletes CCR5$^+$ T cells and monocytes, but is inactive against cells that do not express CCR5. Furthermore, ex vivo the bispecific Ab eliminated >95% of CCR5$^+$ monocytes and T cells from the synovial fluid of patients with arthritis. Also, we designed a fusion protein of the chemokine RANTES and a truncated version of *Pseudomonas* exotoxin A. The fusion protein binds to CCR5 and down-modulates the receptor from the cell surface. The chemokine toxin completely destroyed CCR5$^+$ Chinese hamster ovary cells at a concentration of 10 nM, whereas no cytotoxic effect was detectable against CCR5$^-$ Chinese hamster ovary cells. Both constructs efficiently deplete CCR5-positive cells, appear as useful agents in the treatment of chronic inflammatory diseases, and may help to eradicate HIV-1 by increasing the turnover of latently infected cells. *The Journal of Immunology*, 2001, 166: 2420–2426.

The chemokine receptor CCR5 is a member of a large family of G protein-coupled seven-transmembrane domain receptors that binds the proinflammatory chemokines RANTES, macrophage-inflammatory protein 1α (1), macrophage-inflammatory protein 1β, and monocyte chemotactic protein 2 (1, 2). Chemokines act in concert with adhesion molecules to induce the extravasation of leukocytes and to direct their migration to sites of tissue injury (3). In a variety of chronic inflammatory diseases, an impressive accumulation of CCR5-positive T cells and macrophages is found at the site of inflammation. An accumulation of CCR5$^+$ cells has been demonstrated in several types of arthritis (4, 5), inflammatory renal diseases including transplant rejection (6, 7), multiple sclerosis (8, 9), and inflammatory bowel diseases (10). In contrast, in the peripheral blood of these patients only a minority of T cells and monocytes express CCR5. Therefore, CCR5 appears to be an excellent marker to identify leukocytes that are involved in chronic inflammation. The occurrence of a 32-bp deletion in the CCR5 gene that prevents expression of CCR5 (11) allows study of the pathophysiological role of CCR5 in chronic inflammatory diseases. In patients with rheumatoid arthritis, the frequency of CCR5-deficient (CCR5-Δ32/Δ32)[2] (5) individuals is significantly reduced (12). Moreover, the mean survival of kidney transplants is significantly longer in CCR5-Δ32/Δ32 patients (D. Schlöndorff, personal communication). These results make CCR5 look like a promising target for therapeutic intervention. Furthermore, the predominance of CCR5-positive leukocytes in the diseased tissue in contrast to its rare expression on peripheral blood leukocytes suggests that a specific elimination of CCR5-positive leukocytes may be therapeutically useful by reducing the number of infiltrating cells in chronic inflammation without significantly depleting peripheral blood leukocytes. Eliminating CCR5-positive leukocytes from the inflammatory infiltrate should be of greater therapeutic benefit than simply blocking chemokine receptors of these cells, as they have already infiltrated the tissue.

Besides its role in inflammation, CCR5 is the primary coreceptor for M-tropic HIV-1 strains that predominate early in the course of an infection (13, 14). Transmission of HIV-1 depends on the presence of CCR5, as individuals with a homozygous Δ32 deletion of the CCR5 allele are highly resistant against infection with HIV-1 (11). Although antiretroviral therapy can efficiently suppress replication of HIV-1, complete eradication of HIV has not been achieved to date. The main obstacle appears to be the inactivity of antiretroviral therapy against latently infected cells that can survive for several years and function as an endogenous source for HIV-1 (15). Many of these cells fail to express viral proteins and can evade the immune response. However, the majority of latently infected cells may still express CCR5, as this receptor was necessary for their initial infection. We therefore propose that depletion of CCR5-positive cells should significantly reduce the number of latently infected cells in HIV-1 infection. Other strategies to eliminate HIV-1-infected cells that depend on a specific recognition of viral proteins, e.g., surface-expressed gp120 (16), would be less effective against latently infected cells.

To test these possibilities, we designed two strategies to destroy CCR5-positive cells. First, we constructed a bispecific single-chain Ab that binds with one arm to CCR5 and with the other arm to

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[2] Abbreviations used this paper: CCR5-Δ32/Δ32, homozygous 32-bp deletion in the CCR5 alleles; CHO, Chinese hamster ovary; PE38, truncated version of *Pseudomonas* exotoxin A; CXCR, CXC chemokine receptor.

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CD3 expressed on T lymphocytes. Thereby the bispecific Ab induces a close contact between CCR5-positive target cells and CD3-positive T cells. Cross-linkage of CD3 by the Ab triggers the cytotoxic activity of T lymphocytes and leads to lysis of CCR5-positive cells (Fig. 1). Bispecific Abs were originally developed for oncological therapy to redirect cytotoxic T cells against malignant cells (17). Various types of bispecific Abs have been described so far. Although the chemical cross-linkage of two mAbs and the hybrid-hybridoma technology (18) is hampered by ill-defined protein aggregates and low yields, diabodies and bispecific single-chain Abs have overcome these problems (19–23). In 1995, we described functional expression of bispecific single-chain Abs in Chinese hamster ovary (CHO) (3) cells (20, 24). These Abs are composed of two single-chain Fv fragments that are joined by a flexible linker consisting of glycine and serine molecules. We now propose a new application for bispecific Abs, namely, elimination of infiltrating leukocytes in chronic inflammation. The CCR5-CD3-bispecific Ab would specifically deplete CCR5-positive cells and reduce considerably the number of cells that infiltrate inflamed tissue. T cells and monocytes that are not involved in the inflammation would largely remain unaffected because they rarely express CCR5.

In a second approach to destroy chemokine receptor-positive cells, we designed a fusion protein of the chemokine RANTES and a truncated version of the *Pseudomonas* exotoxin A. Several fusion proteins with a truncated version of *Pseudomonas* exotoxin A have been designed so far. Most of them have been used to target and destroy malignant cells (25). A truncated version of the toxin (PE38) was used for the construct (26), as the full-length protein binds with its first domain to the ubiquitous α_2-macroglobulin receptor and is therefore toxic to most eukaryotic cells (27). To overcome this problem, the first domain of *Pseudomonas* exotoxin A can be replaced by a specific sequence to alter the binding specificity of the toxin (26). We investigated whether a chemokine can serve as ligand sequence on the toxin, direct it to specific targets by binding to the specific chemokine receptor, and induce uptake of the toxin and lysis of chemokine receptor-positive cells. We considered chemokines to efficiently mediate the uptake of the toxin as chemokine receptors are rapidly internalized upon binding of chemokines (28, 29). In this study, we show that in fact the chemokine RANTES fused to PE38 binds to CCR5 and efficiently destroys CCR5-positive target cells. Thus, both the bifunctional CD3-CCR5 Abs and the RANTES-*Pseudomonas* A toxin constructs are promising candidates as therapeutic agents.

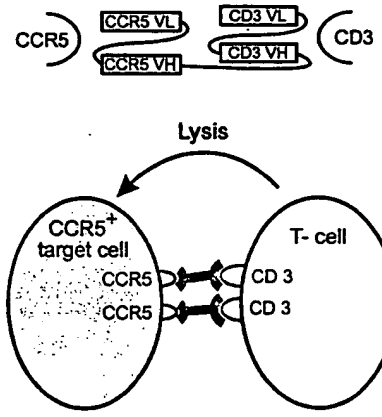

FIGURE 1. Scheme of the bispecific single-chain Ab. The anti-CCR5 single-chain fragment (CCR5 V_L/CCR5 V_H) derived from the hybridoma MC-1 is fused to the N terminus of a single-chain fragment directed against CD3 (CD3 V_H/CD3 V_L). Binding of the bispecific Ab to CD3$^+$ T cells and CCR5-positive target cells results in cross-linkage of CD3, activation of effector T cells, and lysis of CCR5-positive target cells.

Materials and Methods

PBMC, synovial fluid, and cell lines

PBMC were isolated from buffy coats or full blood of healthy donors by Ficoll density gradient centrifugation. Where indicated, PBMC were used from donors with a homozygous 32-bp deletion in the CCR5 allele (CCR5-Δ32/Δ32) preventing surface expression of CCR5. Synovial fluid of patients with arthritis was obtained from diagnostic or therapeutic arthrocentesis and used for the experiments without further preparation. Informed consent was obtained from all patients.

CHO cells were stably transfected with CCR5 or CXC chemokine receptor (CXCR) 4 as described previously (28).

Construction and expression of the bispecific single-chain Ab anti-CCR5-anti-CD3

The light (V_L) and heavy (V_H) variable domains from the anti-CCR5 hybridoma MC-1 were cloned using PCR amplification (30). Reverse transcription was conducted with random hexamer nucleotides and SuperScript reverse transcriptase (Life Technologies, Grand Island, NY). The variable domains were amplified by PCR with Pfu-polymerase, subcloned into the vector PCR-script Amp SK$^+$ (Stratagene, La Jolla, CA), and sequenced. As described previously, the light and heavy variable domains were joined to a single-chain fragment using a $(Gly_4Ser_1)_3$ linker and expressed in the periplasmic space of *Escherichia coli* to test binding of the recombinant protein to CCR5. Subsequently, the DNA sequence of the anti-CCR5 single-chain fragment was subcloned with BsrG1 and BspE1 into an eukaryotic expression vector (pEF-DHFR) that contained a single-chain fragment directed against CD3 with a C-terminally attached tail of six histidine residues (20). The anti-CCR5 and anti-CD3 single-chain fragments were joined by a linker coding for Gly_4Ser_1. The bispecific Ab was expressed in DHFR-deficient CHO cells and purified from the culture supernatant by affinity chromatography on immobilized Ni^{2+} ions (Ni-NTA; Qiagen, Chatsworth, CA).

Construction and expression of RANTES-PE38

A PCR fragment of RANTES, generated with the primers P1 and P2, was subcloned with *StuI* and *SalI* into a vector for periplasmic expression in *E. coli* (20). The restriction site *StuI* had previously been introduced at the 3′ terminus of the OmpA signal sequence. The DNA of a truncated version of Pseudomonas exotoxin A (PE38), kindly provided by I. Pastan (26), was amplified by PCR with Pfu-polymerase using the primers P3 and P4 and subcloned with *Bsp*E1 and *Hin*dIII into the vector that already contained the cDNA of RANTES. Primer P4 also added a tail of 6 histidine residues at the 3′ terminus of PE38. During the periplasmic expression, the OmpA signal sequence is cleaved off such that the recombinant protein starts with the first amino acid of RANTES. The C-terminally attached tail of six histidine residues allowed purification by affinity chromatography on Ni-NTA (Qiagen). List of primers is as follows: P1, 5′-AAAGGCCTC CCCATATTCCTCGGA; P2, 5′-AAAGTCGACTCCGGACATCTCCAAAGA GTTGATGTAC; P3, 5′-AATCCGGAGGCGGCAGCCTGGCCGC; and P4, 5′-GGGAAGCTTAGTGATGGTGATGGTGATGCTTCAGGTCCTCGCGCGG.

FACS analysis and Western blot

Binding of the bispecific single-chain Ab to CHO cells or PBMC was determined by FACS analysis. The cells were incubated with the bispecific Ab for 60 min on ice followed by an Ab against 6xHis (Dianova, Hamburg, Germany) and a PE-conjugated polyclonal rabbit anti-mouse F(ab′)$_2$ fragment (R439; Dako, Hamburg, Germany).

Western blots to detect the bispecific Ab or RANTES-PE38 were stained with the mAb against 6xHis (Dako) and a peroxidase-labeled polyclonal rabbit anti-mouse Ab (P260; Dako). Western blots to detect CCR5 in the cell-free supernatant were performed as previously described (31).

Down-modulation of chemokine receptors

PBMC were incubated for 30 min at 37°C with various concentrations of RANTES or RANTES-PE38 diluted in RPMI 1640 with 10% FCS in a volume of 100 μl. Medium alone was used as control. The cells were then stained on ice for surface CCR5 expression using the mAb MC-1 or medium as negative control followed by the PE-conjugated anti-mouse Ab R439. As shown previously, the presence of RANTES bound to CCR5 does not block the binding of MC-1 to CCR5 (28). The same could be

demonstrated for RANTES-PE38. The FACS analysis was performed on a FACSCalibur (Becton Dickinson, Mountain View, CA) and CellQuest software. Lymphocytes and monocytes were distinguished by their forward and sideward light scatter properties and expression of CD14, CD4, and CD8. Relative surface CCR5 expression was calculated as [mean channel fluorescence (experimental) − mean channel fluorescence (negative control)]/[mean channel fluorescence (medium) − mean channel fluorescence (negative control)].

Depletion of cells with the bispecific anti-CCR5-anti-CD3 Ab and RANTES-PE38

PBMC from CCR5 wild-type or CCR5-deficient (Δ32/Δ32) donors were incubated overnight to induce expression of CCR5 on monocytes. Cultured PBMC or freshly drawn synovial fluid of patients with arthritis were incubated with different concentrations of purified anti-CCR5-anti-CD3-bispecific Abs or medium as control for 20 h. No preactivation of effector T cells was performed. Surviving cells were analyzed on a FACSCalibur and counted.

CHO cells expressing CCR5 or CXCR4 were grown to subconfluence on 24-well culture plates and incubated with different concentrations of purified RANTES-PE38 or medium as control. After 40 h, the adherent and nonadherent cells were recovered and analyzed by FACS to measure the percentage of dead cells. We have previously established that dead (propidium iodide-positive) CHO cells can be identified by their light scatter properties.

Results

Production of a bispecific single-chain Ab directed against CCR5 and CD3

As described in *Materials and Methods*, we amplified the DNA sequences coding for the variable domains of the light (V_L) and heavy chain (V_H) of the CCR5-specific hybridoma MC-1 by RT-PCR. Subsequently, we constructed a single-chain fragment by joining the V_L and V_H sequences with a linker coding for $(Gly_4Ser_1)_3$ and expressed it in the periplasmic space of *E. coli* to test the binding activity of the construct. To obtain the bispecific single-chain Ab, we joined the DNA sequence of the CCR5 Ab fragment to an Ab fragment directed against CD3 using a linker coding for Gly_4Ser_1. The bispecific single-chain Ab was expressed in CHO cells and purified via a C-terminally attached histidine tail with an overall purification yield of ~900 μg/L culture supernatant. SDS-PAGE showed a single band of ~60 kDa under reducing and nonreducing conditions without any detectable proteolysis or degradation of the protein (Fig. 2).

Binding of the bispecific Ab to CCR5 and CD3

Binding of the bispecific Ab to CD3-positive T cells was demonstrated by FACS analysis (Fig. 3). As the bispecific Ab would also bind to CCR5, we performed the analysis with PBMC that lack expression of CCR5 due to a homozygous 32-bp deletion in the CCR5 alleles. The Ab showed good binding to T cells as identified by costaining with Abs against CD4 and CD8 (Fig. 3). In addition,

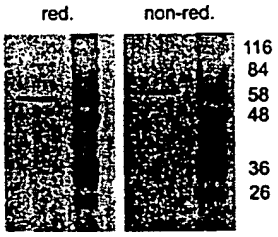

FIGURE 2. SDS-PAGE of the purified bispecific single-chain Ab anti-CCR5-anti-CD3. A single band of ~60 kDa is visible under reducing (*left*) and nonreducing (*right*) conditions. No degradation or proteolysis of the bispecific Ab is detectable.

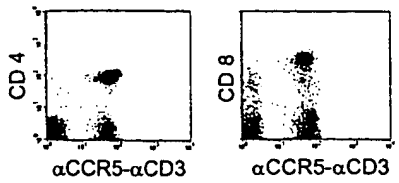

FIGURE 3. Binding of the anti-CCR5-anti-CD3-bispecific Ab to CD3 on CCR5-Δ32/Δ32 lymphocytes. Costaining with CD4 and CD8 demonstrated that the bispecific Ab binds to the subpopulation of $CD4^+/CD8^+$ T cells.

the bispecific Ab competed with the monoclonal CD3 Ab OKT-3 for binding to T cells (data not shown).

Binding of the bispecific Ab to CCR5 was demonstrated on CCR5-overexpressing CHO cells and human monocytes (Fig. 4). The Ab showed excellent binding to CCR5-transfected CHO cells (Fig. 4*A*) and cultured monocytes (Fig. 4*B*), while no binding was detectable on CHO cells transfected with CXCR4 or on cultured monocytes from a donor with a homozygous CCR5-Δ32/Δ32 deletion. Overnight cultivation of monocytes induces expression of CCR5 on wild-type monocytes, while monocytes from donors with a homozygous CCR5-Δ32/Δ32 deletion fail to express CCR5. Moreover, the CCR5 signal detectable with the bispecific Ab on cultured monocytes could be reduced to values below 15% by preincubation of monocytes for 30 min at 37°C with AOP-RANTES, a CCR5 ligand that is known to efficiently induce internalization of CCR5 (data not shown) (28). Preabsorption of the bispecific Ab on $CCR5^+$ CHO cells prevented subsequent binding to CD3 and preabsorption on CCR5-deficient T cells almost completely abolished subsequent binding to CCR5 (data not shown). This further indicates that both specificities of the bispecific Ab are contained in the same molecule.

CCR5-specific depletion of monocytes from cultured PBMC

To test the ability of the anti-CCR5-anti-CD3-bispecific single-chain Ab to deplete CCR5-positive primary cells, we incubated human PBMC with the bispecific Ab (Fig. 5). Before incubation the PBMC were cultured overnight to up-regulate CCR5 expression on monocytes (28, 32). By retargeting cytotoxic T cells, the bispecific Ab depleted the majority of monocytes within 20 h in a concentration-dependent manner (Fig. 5). In contrast to the bispecific Ab, the monovalent single-chain Fv fragments directed

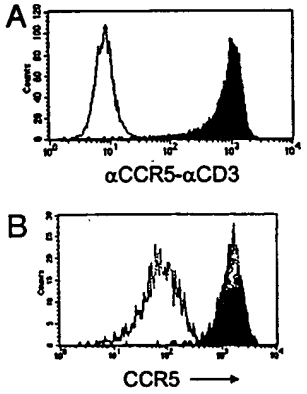

FIGURE 4. Binding of the anti-CCR5-anti-CD3-bispecific Ab to CCR5 on transfected CHO cells (*A*) and cultured monocytes (*B*). CHO cells transfected with CCR5 and monocytes from a CCR5-positive donor are shown in black, while CXCR4-positive CHO cells and monocytes from a CCR5-deficient donor (Δ32/Δ32) served as negative control and are shown in white.

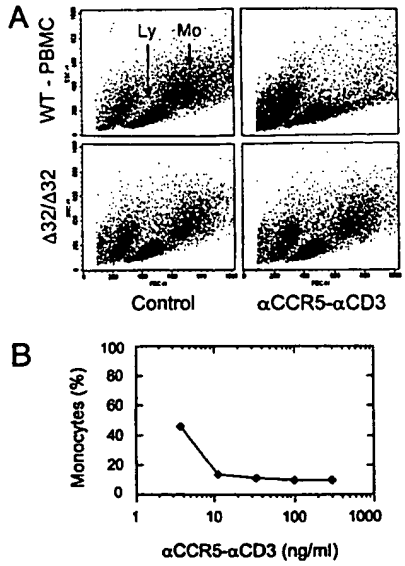

FIGURE 5. Depletion of CCR5-positive monocytes by the bispecific Ab. *A*, CCR5-deficient PBMC (Δ32/Δ32) or wild-type PBMC (WT-PBMC) were cultured overnight and incubated with the bispecific Ab (100 ng/ml) or medium as control for 20 h. Remaining monocytes (Mo) and lymphocytes (Ly) were identified by their light scatter properties in FACS. The CCR5-positive wild-type monocytes were completely depleted by the bispecific Ab, whereas the CCR5-deficient monocytes survived. *B*, Dose response showing depletion of cultured monocytes with various concentrations of the anti-CCR5-anti-CD3-bispecific Ab. More than 90% of the monocytes were depleted at a concentration of 33 ng/ml.

against CCR5 and CD3 used either alone or in combination were not able to deplete CCR5-positive monocytes (data not shown). To verify that the depletion of monocytes with the bispecific Ab was due to their expression of CCR5, we performed the same experiment with PBMC from a donor with a homozygous 32-bp deletion in the CCR5 allele that prevents surface expression of CCR5. No depletion of CCR5-deficient monocytes occurred after 20 h, indicating that the depletion of cells with the bispecific Ab is restricted to monocytes that express CCR5 (Fig. 5*A*). As further control, we preincubated PBMC with an excess of the parental mAb MC-1 or a control Ab and then added the bispecific Ab. Preincubation with MC-1 considerably reduced the depletion of monocytes while the control Ab had no effect (data not shown).

Depletion of monocytes and T lymphocytes from the synovial fluid of patients with arthritis

The bispecific single-chain Ab could potentially be applied to deplete CCR5-positive T cells and monocytes from the inflamed joints of patients with arthritis. We therefore determined the depletion of CCR5-positive cells from the synovial fluid of patients with various types of arthritis. Before each depletion experiment, we confirmed by FACS analysis that the majority of lymphocytes and monocytes in the synovial samples express CCR5, whereas no expression of CCR5 was detectable on granulocytes (data not shown). The synovial fluid was incubated ex vivo with different concentrations of the bispecific Ab immediately after arthrocentesis without prior preparation or washing steps. Using freshly drawn synovial fluid, we aimed at testing the efficacy and stability of the bispecific Ab under conditions resembling most closely the situation in vivo. Within 20 h, the bispecific Ab induced depletion of the majority of lymphocytes and monocytes from the synovial fluid, while granulocytes that do not express CCR5 remained unaffected (Fig. 6).

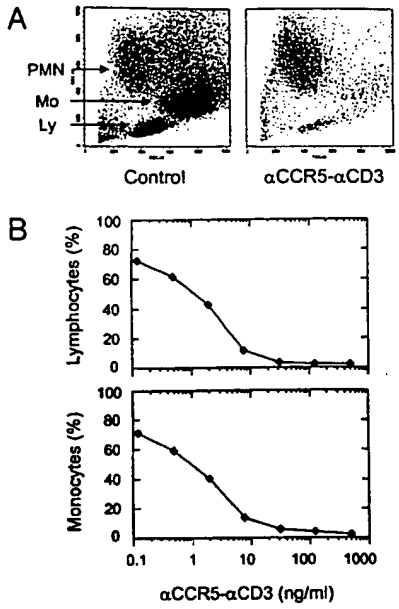

FIGURE 6. The bispecific anti-CCR5-anti-CD3 Ab depletes lymphocytes and monocytes from the synovial fluid of a patient with chronic arthritis. *A*, Freshly draw synovial fluid was incubated with the bispecific Ab (500 ng/ml) or medium as control for 20 h and analyzed by FACS. The bispecific Ab completely depleted the CCR5-positive monocytes and lymphocytes, whereas the CCR5-negative granulocytes (PMN) survived. Consistent with our previous data, all monocytes and lymphocytes in this synovial fluid expressed CCR5, whereas no expression of CCR5 was found on granulocytes (data not shown). *B*, Dose response for the depletion of monocytes and lymphocytes from the synovial fluid with the anti-CCR5-anti-CD3-bispecific Ab. More than 95% of both cell types were depleted at a concentration of 31 ng/ml.

Construction of the chemokine toxin RANTES-PE38

As described in *Materials and Methods*, the DNA sequence of RANTES was fused with the sequence of a truncated version of the *Pseudomonas* exotoxin A (PE38) kindly provided by I. Pastan (26). In a first version of the construct, a Gly-Ser linker was spaced between RANTES and PE38. However, this resulted in a considerable proteolytic degradation of the fusion protein during expression in *E. coli* (data not shown). To stabilize the construct, we removed the linker and the first three amino acids of PE38. The new fusion protein showed no proteolysis during expression in the periplasmic space of *E. coli* as demonstrated by SDS-PAGE and Western blot (Fig. 7).

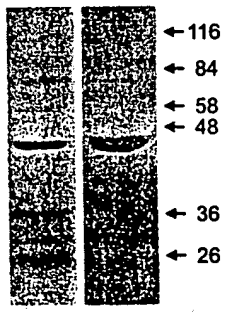

FIGURE 7. SDS-PAGE (*left*) and Western blot (*right*) of the purified protein RANTES-PE38. A distinct band with the expected size of ~46 kDa is visible in the Coomassie-stained SDS-PAGE and Western blot. Most likely our affinity purification is not 100%, which explains the faint bands in the Coomassie stain.

Biologic activity of the RANTES-PE38 construct

The fusion of RANTES to the N terminus of a truncated version of the *Pseudomonas* exotoxin A is supposed to result in specific binding of the construct to cells expressing RANTES receptors such as CCR5, CCR1, and CCR3. Internalization of the chemokine receptors upon binding of the modified toxin would enhance the cellular uptake and cytotoxic activity of the construct. We therefore analyzed whether RANTES-PE38 is able to internalize CCR5 from the surface of primary monocytes and T cells (Fig. 8). Internalization of CCR5 would indicate that the construct is able to bind to CCR5 and that RANTES remains functionally active after fusion to PE38. As shown in Fig. 8, the construct is able to internalize CCR5 from the surface of monocytes and lymphocytes. Compared with unmodified RANTES, the chemokine toxin was somewhat less effective in down-modulating CCR5. To exclude the possibility that RANTES-PE38 increases the shedding of CCR5 from the cell surface and thereby reduces surface expression of CCR5, we quantified by Western blot the amount of CCR5 in the cell-free supernatant after incubation of CCR5-positive CHO cells for 30 min at 37°C with unmodified RANTES, RANTES-PE38, or medium alone. Compared with the medium control, no increased shedding of CCR5 was detectable with either RANTES or RANTES-PE38, indicating that the reduced surface expression is due to internalization of CCR5.

We then analyzed the cytotoxic activity of RANTES-PE38. For that purpose, we incubated CHO cells expressing human CCR5, murine CCR5, and human CXCR4 with various concentrations of the chemokine toxin or medium. No surviving (i.e., adherent) human or murine CCR5-positive CHO cells were detectable by light microscopy after a 40-h incubation with as little as 10 nM RANTES-PE38. In contrast, regular growth and survival was observed when the CCR5-positive cells were incubated with medium or when CXCR4-positive CHO cells were incubated with equal concentrations of the chemokine toxin (data not shown). To quantify the percentage of dead cells, we analyzed the adherent and nonadherent cells by FACS. We have previously established that living and dead CHO cells can be identified by their light scatter properties. As shown in Fig. 9*A*, no cytotoxic effect of RANTES-PE38 was seen on CHO cells expressing CXCR4, whereas CHO cells expressing human CCR5 were completely killed by 10 nM RANTES-PE38. As further control, we preincubated $CCR5^+$ CHO cells with 10 μg/ml unconjugated RANTES and then added the chemokine toxin (Fig. 9*B*). Preincubation of the $CCR5^+$ cells with unconjugated RANTES completely prevented their destruction by RANTES-PE38. RANTES alone did not influence the viability of the cells.

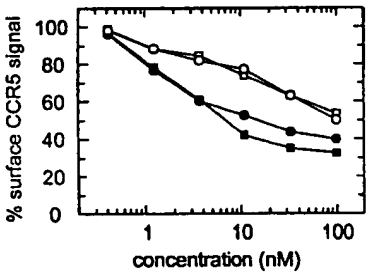

FIGURE 8. Down-modulation of CCR5 from the surface of PBMC by incubation with RANTES-PE38 (○, □) and RANTES (●, ■) for 30 min at 37°C. Surface expression of CCR5 was determined on lymphocytes (□, ■) and monocytes (○,●) and is given as percentage of the medium control. The fusion protein RANTES-PE38 is able to down-modulate CCR5 from the cell surface with a somewhat lower efficiency than unmodified RANTES.

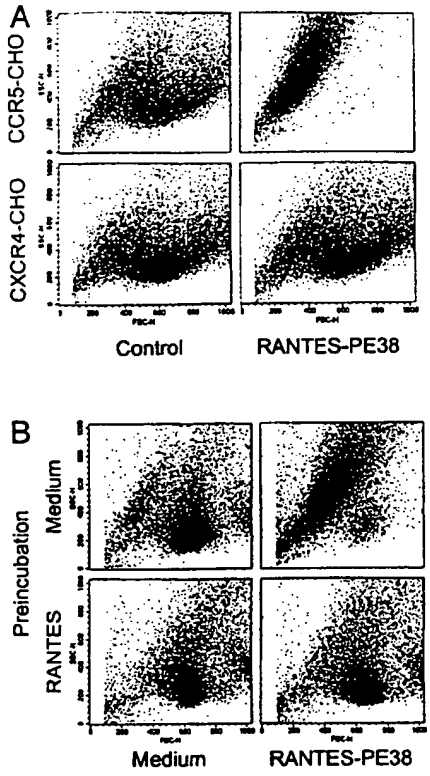

FIGURE 9. Destruction of CCR5-positive CHO cells with the chemokine toxin RANTES-PE38. *A*, CCR5-positive CHO cells and CXCR4-positive CHO cells were incubated for 40 h with the chemokine toxin (10 nM) and analyzed by FACS. Dead cells appear in the *left upper region* of the forward and sideward light scatter plot. RANTES-PE38 completely destroyed the CCR5-positive CHO cells, whereas it had no effect on the CXCR4-positive CHO cells. *B*, The cytotoxic activity of RANTES-PE38 could be completely blocked by addition of unmodified RANTES (10 μg/ml) 60 min before incubation with RANTES-PE38. Preincubation with medium served as control and did not prevent the killing of cells.

These experiments indicate that RANTES-PE 38 is able to internalize CCR5 from the surface of cells and induces depletion of cells expressing the RANTES receptors human CCR5 or murine CCR5. The inactivity of the construct against CXCR4-positive CHO cells demonstrates that the cytotoxic activity of the construct is restricted to cells that express specific chemokine receptors.

Discussion

We propose that a specific depletion of CCR5-positive cells may be therapeutically useful in chronic inflammatory diseases and HIV-1 infection. In chronic inflammation such as rheumatoid arthritis (4, 5), inflammatory renal diseases including transplant rejection (6), multiple sclerosis (8, 9), and inflammatory bowel disease (10), a clear predominance of T cells and monocytes expressing the chemokine receptor CCR5 is found within the affected tissues. In contrast, in the peripheral blood only a minority of T cells and monocytes express CCR5 (4). A depletion of CCR5-positive cells may therefore considerably reduce the infiltrate in the inflamed tissue while other leukocytes would remain largely unaffected. Also, identification of latently HIV-infected cells by CCR5 and their specific depletion could be a clinical application of the bispecific Abs and chemokine toxins.

We present two possibilities to deplete CCR5-positive cells. First, we describe a bispecific single-chain Ab that simultaneously binds to CCR5 and CD3 and thereby redirects T effector cells

against CCR5-positive target cells (Fig. 1). A precondition for the efficacy of this bispecific Ab is the presence of T effector cells. In rheumatoid arthritis, T cells are enriched in the synovial fluid and tissue and their percentage further increases with disease duration. Since most of these T cells are CCR5 positive, they would deplete each other as shown for the synovial fluids of patients with arthritis (Fig. 6). Many of the infiltrating T cells potentially recognize autoantigens and therefore perpetuate the disease process. In chronic glomerulonephritis, interstitial nephritis, and transplant rejection, CCR5-positive T cells are abundantly present in areas of interstitial infiltration and their number correlates with the degree of renal insufficiency (6). Apart from T cells, CCR5 is expressed by the majority of monocytes/macrophages within the inflamed tissue. Monocytes/macrophages are responsible for destruction of inflamed tissue. The depletion of infiltrating monocytes/macrophages may therefore considerably reduce the tissue damage, e.g., joint destruction in rheumatoid arthritis. Moreover, CCR5 is highly expressed on dendritic cells (33) that are found in the synovial fluid and synovial tissue in rheumatoid arthritis and are thought to play a major role for initiation and perpetuation of the inflammatory process (34).

A specific depletion of CCR5-positive cells appears to have several advantages over conventional immunosuppressive or immunomodulatory therapy: 1) Depletion of infiltrating cells would only take a couple of hours, while immunomodulatory agents usually need several weeks for their onset of action. 2) Conventional treatments only suppress the activity of infiltrating cells which leads to a rapid relapse after termination of the therapy. In contrast the bispecific Ab would eliminate the infiltrating cells and may therefore exert a prolonged benefit. 3) Elimination of CCR5-positive leukocytes is supposed to be well tolerated as CCR5-positive cells are enriched in the inflamed tissue and only rarely encountered in the peripheral blood. A local application of bispecific Abs, e.g., intra-articular injection in arthritis, would further reduce potential side effects.

Chemokine toxins significantly differ from bispecific Abs in their mechanism of depletion. Although bispecific Abs depend on T effector cells to deplete other cells, the mere binding and internalization of chemokine toxins is sufficient to induce cell death. Binding and internalization of chemokine toxins depends on the expression of appropriate chemokine receptors. In many cases, chemokines bind to more than one receptor, which would result in a more extensive destruction of cells. The chemokine toxin RANTES-PE38 binds to CCR5 and eliminates CCR5-positive CHO cells. However, we assume that the construct would also recognize and destroy CCR1- or CCR3-positive cells since RANTES also binds to these receptors (35). The main advantage of chemokine toxins is their ease of production, as the chemokine moiety can be rapidly exchanged by one cloning step. For most receptors, one would also be able to select a chemokine that only binds to one receptor. Chemokine toxins would therefore be an ideal tool to study depletion of selected subtypes of cells in various animal models of inflammation.

The expression of chemokine receptors on leukocytes is associated with certain types of an immune response. Recently, it was described that the receptors CCR5 and CXCR3 are primarily expressed on TH-1 cells, while CCR3, CCR4, and CCR8 are mainly found on TH-2 cells (36–39). Depletion of chemokine receptor-positive cells may therefore induce an immune deviation from TH-2 to TH-1 or vice versa. Depletion of CCR3-positive cells may be of benefit in allergic diseases, as CCR3 is not only expressed on TH-2 cells but also on eosinophils and basophils (40, 41).

Apart from chronic inflammatory diseases, the depletion of CCR5-positive cells could also be useful for the treatment of HIV-1 infection to reduce the number of latently infected cells. Early in the course of an HIV infection, preferentially CCR5-positive cells are infected with HIV-1 (14). A small fraction of these cells, mainly $CD4^+$ T cells, macrophages, and potentially dendritic cells, become latently infected and function as an endogenous source for HIV-1 (15). Latently infected cells can survive for several years and are thought to be responsible for the failure to completely eradicate HIV-1 despite prolonged highly active antiretroviral therapy. We therefore propose that depletion of $CCR5^+$ cells would shorten the half-life of latently infected cells and could help to eradicate HIV-1 in combination with highly active antiretroviral therapy. Latently infected cells cannot be identified by surface expression of viral proteins, as little viral genes are expressed when the virus is dormant. CCR5 however would still be expressed on latently infected cells as it was necessary for initial infection.

Specific depletion of chemokine receptor-positive cells can be achieved with bispecific Abs and chemokine toxins and may represent a new strategy in the treatment of chronic inflammatory diseases and HIV-1.

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Immunology

CCR5$^+$ and CXCR3$^+$ T cells are increased in multiple sclerosis and their ligands MIP-1α and IP-10 are expressed in demyelinating brain lesions

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ABSTRACT **Multiple sclerosis (MS) is a T cell-dependent chronic inflammatory disease of the central nervous system. The role of chemokines in MS and its different stages is uncertain. Recent data suggest a bias in expression of chemokine receptors by Th1 vs. Th2 cells; human Th1 clones express CXCR3 and CCR5 and Th2 clones express CCR3 and CCR4. Chemokine receptors expressed by Th1 cells may be important in MS, as increased interferon-γ (IFN-γ) precedes clinical attacks, and IFN-γ injection induces disease exacerbations. We found CXCR3$^+$ T cells increased in blood of relapsing-remitting MS, and both CCR5$^+$ and CXCR3$^+$ T cells increased in progressive MS compared with controls. Furthermore, peripheral blood CCR5$^+$ T cells secreted high levels of IFN-γ. In the brain, the CCR5 ligand, MIP-1α, was strongly associated with microglia/macrophages, and the CXCR3 ligand, IP-10, was expressed by astrocytes in MS lesions but not unaffected white matter of control or MS subjects. Areas of plaque formation were infiltrated by CCR5-expressing and, to a lesser extent, CXCR3-expressing cells; Interleukin (IL)-18 and IFN-γ were expressed in demyelinating lesions. No leukocyte expression of CCR3, CCR4, or six other chemokines, or anti-inflammatory cytokines IL-5, IL-10, IL-13, and transforming growth factor-β was observed. Thus, chemokine receptor expression may be used for immunologic staging of MS and potentially for other chronic autoimmune/inflammatory processes such as rheumatoid arthritis, autoimmune diabetes, or chronic transplant rejection. Furthermore, these results provide a rationale for the use of agents that block CCR5 and/or CXCR3 as a therapeutic approach in the treatment of MS.**

Chemokines, chemotactic cytokines that mediate the attraction of leukocytes to tissues, are essential for inflammatory responses. More than 40 chemokines and 10 chemokine receptors are known, as recently reviewed (1). Recent data suggest a bias in expression of selected chemokine receptors by Th1 cells compared with Th2 cells. In particular, human Th1 clones express CXCR3 (receptor for IP-10 and Mig) and CCR5 (receptor for MIP-1α, MIP-1β, and RANTES), whereas Th2 cells express CCR3 (receptor for eotaxin, RANTES, MCP-3, MCP-4), CCR4 (receptor for TARC and MDC) (2–4) and CCR8 (receptor for I-309) (5).

Multiple sclerosis (MS) is a T cell-dependent chronic inflammatory disease of the central nervous system with a likely autoimmune etiology (6). A central mechanism in the pathogenesis of MS is the organ-specific traffic of T cells into the brain. Though it is known that activated T cells can cross the blood–brain barrier, the mechanisms by which activated T cells are recruited and remain in the brain, and whether chemokines are involved in the pathogenesis of MS, are unknown. Chemokine receptors that are expressed by Th1 cells may be especially important, as increased production of interferon-γ (IFN-γ) precedes clinical attacks (7, 8) and injection of MS patients with recombinant IFN-γ induced exacerbations of the disease (9). In addition, MS involves different stages, usually beginning with a relapsing–remitting phase and later, a progressive form. Immune factors associated with different stages of the disease are not well understood. We studied chemokine receptor expression by mononuclear cells in MS. We found that the numbers of CXCR3$^+$ T cells were increased in relapsing– remitting MS and that both CCR5$^+$ and CXCR3$^+$ T cells were increased in peripheral blood of progressive MS patients compared with healthy controls. In addition, their ligands MIP-1α and IP-10 were strongly associated with microglia/macrophages and astrocytes, respectively, in the MS lesions but not in the control white matter areas of postmortem central nervous system (CNS) samples. Additionally, CCR5$^+$ T cells isolated from peripheral blood of progressive MS patients secreted IFN-γ at a high level. Hence, the selective chemotaxis of IFN-γ-producing CCR5$^+$ T cells into the CNS may be an important mechanism in the pathogenesis of MS, and differential chemokine receptor expression may be associated with different stages of the disease.

MATERIALS AND METHODS

Subjects. MS patients from the outpatient MS clinic of the Brigham and Women's Hospital were studied. Relapsing–remitting patients (n = 14, average age = 40 ± 8 years) had an average expanded disability status (EDSS) of 1.1 ± 0.8, and chronic progressive MS patients (n = 20, average age = 47 ± 6 years) had an EDSS of 5.1 ± 1.9. A disability of 6 or greater involves use of a cane or other support. Patients had not received immunosuppressive therapy in the past, or steroid treatment in the 6 months prior to blood drawing. The control group consisted of healthy subjects (n = 20, average age = 48 ± 8 years). The number of patients used for each individual experiment is given in the corresponding table or figure legend.

Cell Separation. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by centrifugation over Ficoll–Hypaque (Pharmacia LKB Biotechnology). Cells were resuspended (10^6 cells per ml) in RPMI medium 1640 supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 25 mM Hepes buffer, 50 units/ml penicillin, and 50 μg/ml streptomycin (all from BioWhittaker). T cells

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Abbreviations: CNS, central nervous system; MS, multiple sclerosis; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin.

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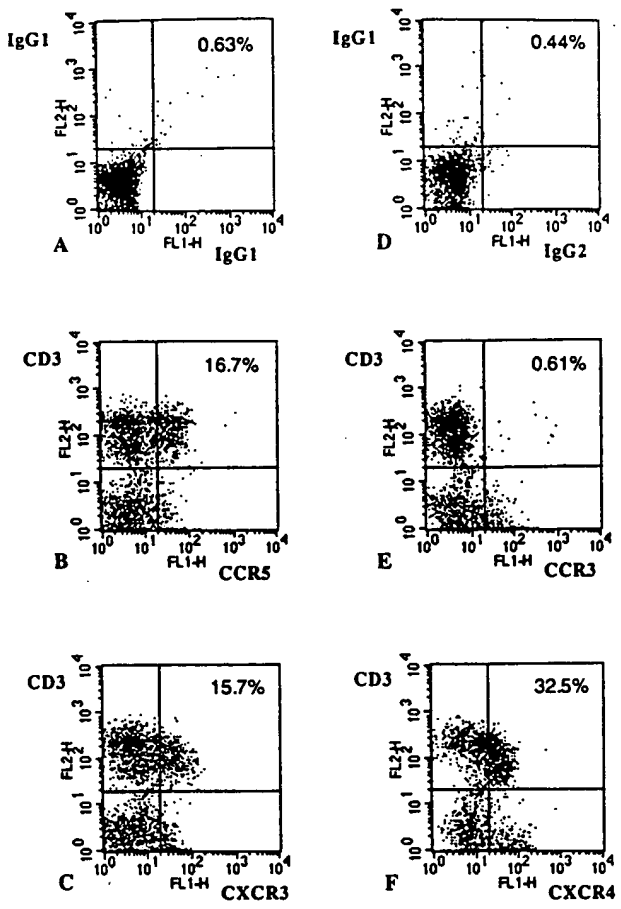

FIG. 1. Separated PBMC from a progressive MS patient were stained with mAb and isotype control, as described in the text. Two-color flow cytometry analysis of 1×10^4 cells from each sample was performed. The percentage of double-positive cells from the lymphocyte populations analyzed is shown. *A* shows staining with an isotype control (mouse IgG1-FITC and mouse IgG1-PE) that is the control for *B* ($CD3^+$ $CCR5^+$ cells) and *C* ($CD3^+$ $CXCR3^+$ cells). *D* represents staining with an isotype control (mouse IgG2a-FITC and mouse IgG1-PE) that is the control for *E* ($CD3^+$ $CCR3^+$ cells) and *F* ($CD3^+$ $CXCR4^+$ cells).

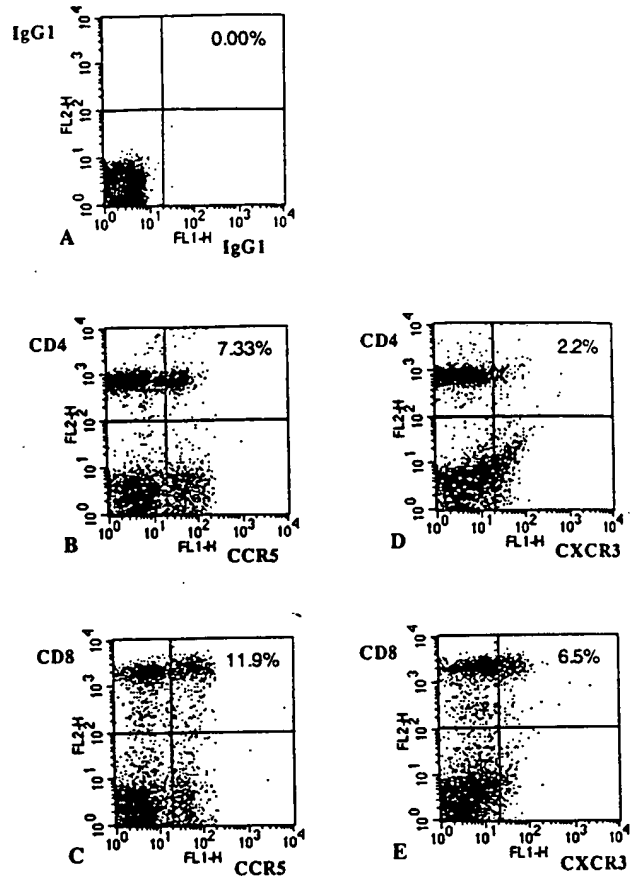

FIG. 2. PBMC from a progressive MS patient were stained with mAb and isotype-matched control Ig, as described in the text. Two-color flow cytometry analysis of 1×10^4 cells from each sample was performed. The percentage of double-positive cells from the lymphocyte populations analyzed is shown. *A* shows staining with an isotype control (mouse IgG1-FITC and mouse IgG1-PE) that is the control for *B* ($CD4^+$ $CCR5^-$ cells), *C* ($CD8^+$ $CCR5^+$ cells), *D* ($CD4^+$ $CXCR3^+$ cells), and *E* ($CD8^+$ $CXCR3^+$ cells).

were separated from PBMC by negative depletion of non-T cells with a human T cell enrichment column (R & D Systems), according to the manufacturer's instructions. T cells were separated into CCR5-depleted ($CCR5^-$) and CCR5-enriched ($CCR5^+$) T cells by using anti-CCR5 mAb (10 μg/ml), goat anti-mouse IgG-coated magnetic microbeads, and a type MS Separation column from Miltenyi Biotec (Auburn, CA), according to the manufacturer's recommendations.

Flow Cytometry. Unlabeled mouse mAbs directed against human CCR3 mAb (7B11, IgG2a), CCR5 (2D7, IgG1), CCR6 (11A9, IgG1), and CXCR3 mAb (1C6, IgG1) were obtained from LeukoSite (Cambridge, MA); anti-CXCR4 mAb (1G5, IgG2a) was purchased from PharMingen (San Diego); and anti-human CD28 mAb (EX5–3D10, IgG2a) was provided by Ed Greenfield (Dana–Farber Cancer Institute, Boston). Control unlabeled mouse IgG1, IgG2a, fluorescein isothiocyanate (FITC)-conjugated polyclonal anti-mouse Ig, phycoerythrin (PE)-conjugated anti-human CD3 mAb (IgG1), PE-anti-human CD4 mAb (IgG1), PE-anti-human CD8 mAb (IgG1), and isotype control PE-IgG1 were purchased from PharMingen. Two-color flow cytometry was performed by incubating 2×10^5 cells in RPMI medium 1640 plus 2% fetal bovine serum with unlabeled mAb (10 μg/ml), followed by FITC-goat anti-mouse Ig (5 μg/ml), and the appropriate directly conjugated mAb(s). Each incubation (20 min, 4°C) was followed with two washes. Unlabeled and labeled appropriate control mouse Ig of identical isotype were used in control samples. Flow cytometric analysis of 1×10^4 cells from each sample was performed on a FACSort flow cytometer (Becton Dickinson) according to standard procedures.

T Cell Activation and Cytokine Assay. Isolated T cells (1×10^5 cells per well) were added to the wells of a 96-well flat-bottom plate (final volume of 0.2 ml) containing immobilized anti-CD3 (OKT3, IgG2a, 1 μg/ml) and anti-CD28 (EX5–3D10, IgG2a, 1 μg/ml) or immobilized control mouse IgG2a. Culture supernatants were collected after incubation for 20 hr (37°C, 5% CO_2). Assays of IFN-γ, interleukin (IL)-4, and IL-5 in culture supernatants were performed by using a cytokine capture ELISA protocol from PharMingen. Components included unlabeled, and corresponding biotinylated, mouse mAbs to human IFN-γ, IL-4, and IL-5, plus recombinant human IFN-γ (GIBCO/BRL), IL-4 (Sigma), and IL-5 (PharMingen) standards. Assay sensitivities were 32 pg/ml (IFN-γ) and 8 pg/ml (IL-4, IL-5).

Human Brain Immunopathology. Frozen brain specimens were obtained from the Rocky Mountain Multiple Sclerosis Center Tissue Bank, Englewood, CO. Samples were collected from cadavers within 6 hr post mortem and snap-frozen in liquid nitrogen. There were five specimens from patients (45 ± 8 years) with active MS in which the tissues were dissected to encompass an area of MS plaque and adjacent normal-

Table 1. Expression of chemokine receptors by peripheral blood T cells in MS patients

Receptors	Double-positive cells, %		
	Controls	RR MS	Progressive MS
$CD3^-$ $CCR3^+$	1.4 ± 0.3 (20)	2.0 ± 0.6 (14)	2.1 ± 0.3 (20)
$CD3^-$ $CCR4^+$	9.0 ± 1.1 (6)	7.1 ± 0.6 (14)	9.6 ± 2.7 (7)
$CD3^-$ $CCR5^+$	7.9 ± 0.8 (20)	9.9 ± 1.3 (14)	13.1 ± 1.4** (20)
$CD3^-$ $CCR6^+$	8.6 ± 1.3 (9)	Not tested	9.2 ± 0.7 (10)
$CD3^-$ $CXCR3^+$	9.1 ± 1.1 (20)	14.4 ± 1.9* (14)	14.8 ± 1.5** (20)
$CD3^-$ $CXCR4^+$	47.1 ± 8.1 (9)	Not tested	30.9 ± 5.3 (10)

PBMC from control healthy donors, relapsing–remitting (RR) MS, and progressive MS patients were separated and a three-step staining procedure, with mouse anti-chemokine receptor mAb followed by FITC-conjugated goat anti-mouse IgG and then PE-labeled anti-CD3 mAb, was performed as described in the text. Two-color flow cytometry was performed, and percentage of double-positive cells was analyzed and presented as mean ± SEM, with the number of patients in parentheses. *, $P < 0.05$ vs. controls; **, $P < 0.005$ vs. controls.

appearing white matter, plus corresponding white matter; five patients (44 ± 9 years) with inactive MS who died from other causes; and five non-MS patients (68 ± 21 years) with normal-appearing brain tissues as controls. Cryostat sections were initially stained by hematoxylin and eosin, and luxol fast blue, and appropriate areas were chosen for evaluation by immunohistology using an avidin–biotin complex method (10). After overnight incubation with primary mAbs and developing reagents, sections were incubated with diaminobenzidine, counterstained with hematoxylin, and evaluated independently by two pathologists (J.E.B. and W.W.H.). Primary mAbs against human chemokines and chemokines receptors were prepared at LeukoSite, mAbs to cell markers were obtained from Dako, and anti-cytokine mAbs were from R & D. These included mAbs to the human chemokine receptors, CCR1 (2D4), CCR2 (1D9), CCR3 (7B11), CCR4 (1G1), CCR5 (5C7), CCR6 (11A9), and CXCR3 (1C6); the chemokines eotaxin (9G3), eotaxin-2 (10G12), IP-10 (6G10), Mig (4G10), ITAC (9E9), MIP-1α (11A3), MIP-3a (6A12), MCP-1 (10F7), MCP-2 (2D5), MCP-3 (9H11), and TARC (208); markers for all leukocytes (CD45, PD7/26), T cells (CD2, MT910), and mononuclear phagocytes (CD68, EBM11); and mAbs to human IFN-γ (MAB-285), IL-4 (MAB204), IL-5 (MAB205), IL-10 (MAB217), IL-13 (MAB213), IL-18 (MAB318), and transforming growth factor-β1 (MAB240), plus isotype-matched control mAbs.

Statistical Analysis. Results are presented as mean ± SEM or mean ± SD for each group. Statistical significance was calculated by using Student's *t* test.

Table 3. CNS expression of chemokines and their receptors in MS

Marker	Normal brain or inactive MS	Active MS
CCR1	Negative	Macrophages/microglia
CCR2	Negative	Occasional macrophages/microglia
CCR5	Microglia, some neurons	Marked up-regulation (mononuclear cells)
CXCR3	Negative	Lymphocytes
MIP-1α	Negative	Macrophages/microglia
MCP-1	Astrocytes	Up-regulated (astrocytes/blood vessels)
IP-10	Astrocytes	Marked up-regulation (same cells)
Mig	Negative	Focal macrophages/microglia
IFN-γ	Negative	Lymphocytes
IL-18	Negative	Focal macrophages/microglia

Based upon assessment of serial sections of brain tissue from five patients with active disease vs. five patients with inactive disease and five normal brain samples; no significant inflammatory cell labeling was seen with mAbs to CCR3, CCR4, or CCR6; the chemokines eotaxin, eotaxin-2, ITAC, MCP-2, MCP-3, MIP-3α, or TARC; or the cytokines IL-4, IL-10, IL-13, or transforming growth factor-β.

RESULTS AND DISCUSSION

Production of IFN-γ, a cytokine that is a hallmark of Th1 type immune responses, is important to the pathogenesis of MS, as increased production of IFN-γ precedes clinical attacks (7, 8), and injection of MS patients with recombinant IFN-γ induces exacerbations of the disease (9). Activated blood mononuclear cells are known to produce significantly higher levels of IFN-γ in progressive MS (11, 12). Furthermore, active lesions in the CNS of MS patients are characterized by mononuclear cell infiltrates including both $CD4^+$ and $CD8^+$ T cells and macrophages (13) and are associated with increased IFN-γ expression (14). However, little is known about the immune basis for selective traffic of IFN-γ-secreting T cells into the CNS or the expression of chemokine of different chemokine receptors in different stages of the disease. Since expression of chemokine receptors by particular T cells subsets may play a role in this selective T cell migration and immune activation and differentiation, we studied chemokine receptor expression by peripheral blood cells from MS patients with relapsing–remitting or chronic progressive forms of the disease, as well as healthy individuals.

$CCR5^+$ and $CXCR3^+$ T cells were identified by flow cytometry. As shown in Fig. 1, CCR5, CXCR3, and CXCR4 were expressed primarily by $CD3^+$ cells, whereas CCR3 expression was associated primarily with non-T cells. As shown in Fig. 2, CCR5 and CXCR3 were expressed by both $CD4^+$ and $CD8^+$ T cells. We then compared the levels of T cells expressing chemokine receptors in peripheral blood samples

Table 2. IFN-γ production by T cell subsets in progressive MS patients

	IFN-γ production by CCR5 cell subsets					IFN-γ production by CXCR3 cell subsets				
	Whole T cells	$CCR5^-$ T cells		$CCR5^+$ T cells		Whole T cells	$CXCR3^-$ T cells		$CXCR3^+$ T cells	
Exp.	pg/ml	pg/ml	%	pg/ml	%	pg/ml	pg/ml	%	pg/ml	%
1	3,375	1,407	42	7,658	224	3,375	3,006	89	2,928	87
2	2,479	1,526	62	2,258	91	2,479	1,474	59	1,474	59
3	1,203	896	74	5,144	428	1,203	282	23	1,625	135
4	1,001	250	25	1,713	171	1,001	425	42	2,935	293
5	3,735	1,112	30	6,572	176		ND		ND	
Mean	2,359 ± 553	31,038 ± 226	47 ± 9*	4,669 ± 1,169	218 ± 57**	2,015 ± 559	1,297 ± 629	53 ± 14***	2,241 ± 400	144 ± 52

T cells from five progressive MS patients were isolated, separated into T cells subsets, and activated with immobilized anti-CD3 plus anti-CD28 mAb. Cell supernatants were collected at 20 h and IFN-γ, IL-4, IL-5 production were measured by ELISA. IL-4 production by purified T cells in all experiments was less than 40 pg/ml. IL-5 production by purified T cells was tested in experiments 2, 3, and 4 and was less that 16 pg/ml. Since donors had different background levels of IFN-γ production prior to T cell subset separation, IFN-γ production for separated subsets is also expressed as a percentage of IFN-γ production by the subset compared to the whole T cell population [(separated subset/whole T cells) × 100%]. ND, not done. *, $P < 0.001$ vs. whole T cells; **, $P < 0.02$ vs. $CCR5^-$ T cells; ***, $P < 0.02$ vs. whole T cells.

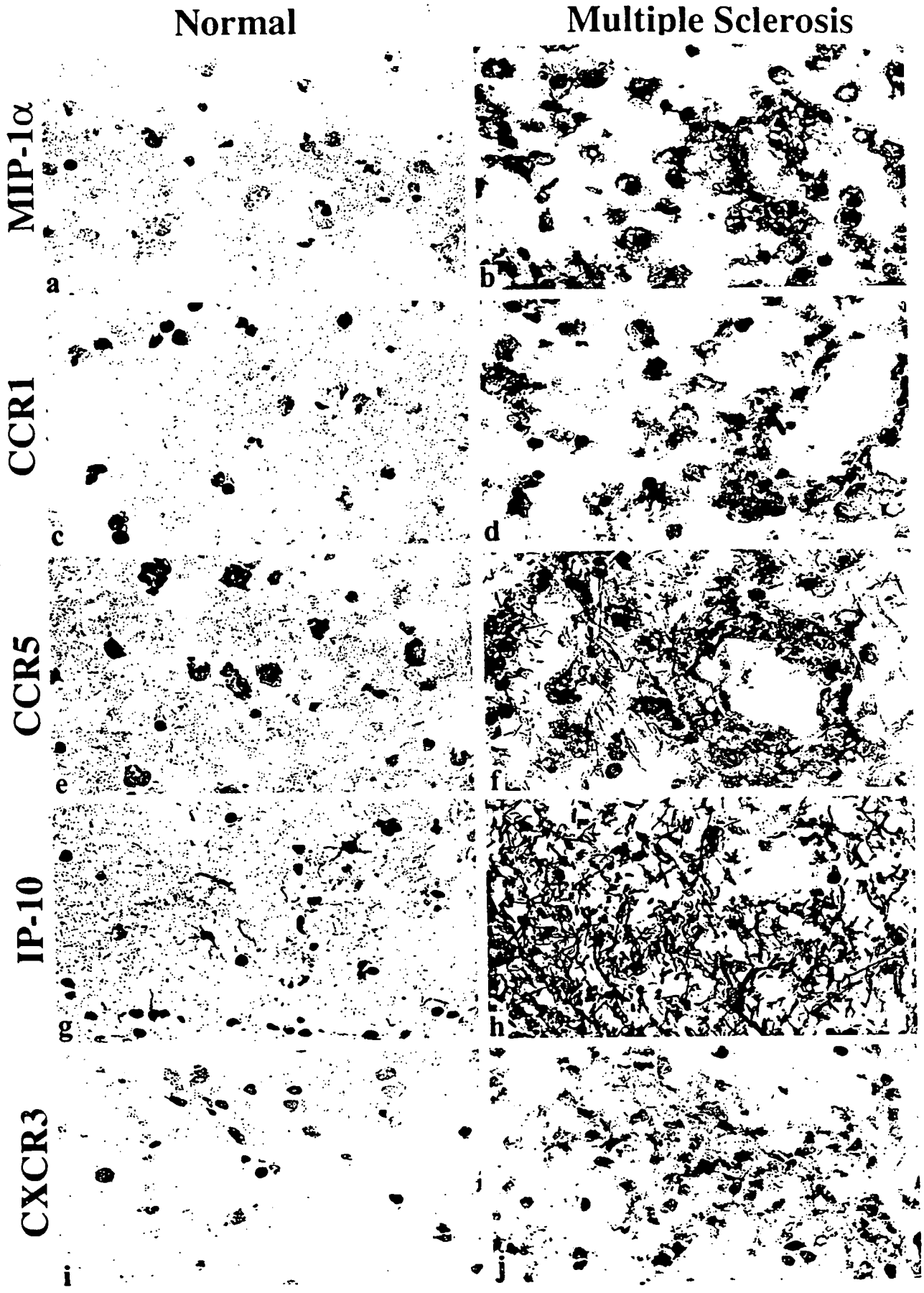

FIG. 3. (*Legend appears at the bottom of the opposite page.*)

from a series of healthy controls ($n = 20$), and patients with relapsing–remitting ($n = 14$) or progressive MS ($n = 20$) (Table 1). We tested chemokine receptors associated with Th1 cells, CCR5 and CXCR3, as well as testing CCR3, a chemokine receptor associated with Th2 cells. CCR6 and CXCR4 served as control chemokine receptors because their expression is not selectively linked with either Th1 or Th2 subset (2–4). Both $CD3^+$ $CCR5^+$ and $CD3^+$ $CXCR3^+$ cells were elevated in progressive MS patients ($P < 0.005$), whereas the percentage of $CXCR3^+$ T cells was increased in relapsing-remitting MS ($P < 0.05$) compared with controls. In contrast, the numbers of $CXCR4^+$, $CCR3^+$, and $CCR6^+$ T cells were not different between MS patients and controls. We found no increase in $CD3^+$ cells expressing CCR4 (associated with the Th2 cells) in chronic progressive MS patients (9.6% ± 2.7%), as compared with control subjects (9.0% ± 1.1%) or relapsing–remitting patients (7.1% ± 0.6%).

We (12) and others (11) have observed that activated peripheral blood T cells from patients with progressive MS produce high levels of IFN-γ, a cytokine characteristic of Th1 lymphocytes. Moreover, recent studies with human T cell clones suggest that CCR5 or CXCR3 chemokine receptors may be markers for Th1 cells, and that CCR3, CCR4, and CCR8 are corresponding markers for Th2 cells (2–5). We therefore investigated whether the expression of CCR5 and CXCR3 receptors by peripheral blood T cells of patients with progressive MS was associated with increased production of IFN-γ. T cells from patients with progressive MS were sorted into $CCR5^+$, $CCR5^-$, $CXCR3^+$, and $CXCR3^-$ T cell subsets and were stimulated with immobilized anti-CD3 and anti-CD28 mAbs. The results from five individuals are presented in Table 2. In five of five experiments, $CCR5^+$ T cells produced increased levels of IFN-γ compared with $CCR5^-$ T cells (4,669 ± 1,169 pg/ml vs. 1,038 ± 226 pg/ml, $P < 0.02$). There was also an increase of IFN-γ secretion in $CXCR3^+$ T cells in two of four patients tested (Table 2). These studies indicate that progressive MS is associated with an increase in circulating activated T cells expressing CCR5 that produce IFN-γ. To assess the possible pathophysiological significance for active disease within the CNS, we undertook immunopathologic evaluation of MS and control brain samples.

Samples of brain from patients with active MS ($n = 5$), inactive MS ($n = 5$), and no MS ($n = 5$) were examined. All samples consisted primarily of white matter. In the active MS brain specimens, extensive areas of the white matter were infiltrated with large numbers of macrophages/microglia, occasional lymphocytes, and scattered neutrophils. The infiltrating cells often formed linear aggregates parallel to swollen axons, and in some areas, the white matter was completely effaced, with only massive numbers of foamy gitter cells and tangled astrocyte processes remaining. Gemistocytic astrocytes were prominent along the periphery of lesions. In some brain specimens, the leukocyte infiltrate extended into the gray matter, resulting in neuronal necrosis.

Immunohistologic studies of serial brain sections allowed analysis of expression of the chemokine receptor/chemokine pairs CCR1/MIP-1α, CCR5/MIP-1α, CXCR3/IP-10/Mig, and CCR2/MCP-1 (Table 3, Fig. 3). Macrophages/microglia in areas of severe inflammation and necrosis were intensely immunoreactive for CCR1, CCR5, and the ligand for both receptors, MIP-1α. Expression of CXCR3 was restricted to small numbers of lymphocytes, which were also $CCR5^+$; these cells were typically detected adjacent to blood vessels of inflamed brain specimens. Perivascular and plaque-associated lymphocytes showed cytoplasmic staining for IFN-γ, and macrophages in these areas expressed the potent IFN-γ-inducing factor, IL-18. Hence, multiple feedback and amplification loops are present within MS lesions, including, in the example of leukocytes alone, $CCR5^+$ IFN-γ-producing T cells, plus the chemokine, IP-10, which is induced by IFN-γ, and corresponding IP-10-responsive $CXCR3^+$ mononuclear cells. These findings contrasted with the lack of leukocyte expression of other chemokine receptor/chemokine pairs, including CCR3/eotaxin/eotaxin-2, CCR4/TARC, and CCR6/MIP-3α.

In addition to leukocytes, astrocytes and vascular cells showed chemokine or chemokine receptor expression. Thus, as with macrophages, astrocytes in regions of inflammation showed dense expression of CCR5, and astrocytes also showed cytoplasmic labeling for the ligands MCP-1 and IP-10. Vascular smooth muscle and endothelium in inflamed brain specimens were immunoreactive for MCP-1, as compared with normal brain specimens, suggesting up-regulation. Negligible CNS expression of the MCP-1 receptor, CCR2, or additional ligands, MCP-2 and MCP-3, was observed, and the results of staining of the available areas of white matter from brains of patients with inactive MS or non-MS patients were similar; overall findings of active MS vs. control samples are summarized in Table 3.

In the present study, we observed increased expression of $CXCR3^-$ T cells in relapsing–remitting patients and of $CXCR3^-$ and $CCR5^-$ T cells in progressive patients. Earlier studies from our group suggest that chronic activation of the immune system occurs when a patient changes from the relapsing–remitting to the progressive stage, as measured by IFN-γ and IL-12 production (12). In the present report we have found that this change in the stage of MS also appears to be linked to chemokine receptor expression. Interestingly, relapsing–remitting MS has been reported in patients with a mutation in the CCR5 gene (15), but the effect of this mutation on progressive MS is unknown. We have also noted an increased number of CD40 ligand (CD40L)-expressing T cells in the peripheral blood of progressive MS patients (12), and $CD40L^+$ T cells have been identified in direct contact with $CD40^+$ macrophages within MS lesions (16). This interaction may be particularly important in the context of MS, because $CD40L^+$ T cells can induce production of MIP-1α, MIP-1β, RANTES, and MCP-1 by $CD40^+$ macrophages (17) and thus may contribute to the chemokine induction we observed in active MS brain lesions.

Since IFN-γ has been reported to be elevated in MS, we tested whether the elevated expression of CXCR3 and CCR5 was related to IFN-γ. We carried out experiments in which we added IFN-γ (10 ng/ml) or IL-12 (1 ng/ml) to PBMC and measured chemokine receptor expression. We found no increase of expression in MS patients after culture with IFN-γ or IL-12, but rather a decrease in expression in the range of 5–40%, depending on the donor ($n = 4$). Similar reductions were observed in healthy controls. These results suggest that although cells expressing CCR5 produce more IFN-γ in MS, the IFN-γ itself does not appear to be directly responsible for the increased expression of this Th1 type chemokine receptor.

FIG. 3. Immunopathology of progressive MS illustrating chemokine and chemokine receptor expression within the CNS in an area of demyelination (*Right*). *Left* micrographs show the very limited expression of these proteins in normal brain, with focal expression of CCR5 by neurons, and IP-10 expression by astrocytes; only minor expression, comparable to normal brain, was seen in the uninvolved tissues of MS patients with inactive disease (not shown). In contrast, areas of demyelination showed dense surface staining of macrophages for MIP-1α (*a* and *b*). Macrophages and small numbers of lymphocytes in these areas showed corresponding expression of CCR1 (*c* and *d*) and CCR5 (*e* and *f*). Lesions contained dense IP-10 expression, associated with astrocytes and their tangled processes (*g* and *h*), plus small numbers of $CXCR3^+$ lymphocytes (*i* and *j*). (Cryostat sections, hematoxylin counterstain, ×100.)

The linkage of CCR5 expression and IFN-γ production thus appears related to the chemotaxis of IFN-γ-producing Th1 cells to sites of inflammation rather than the induction of CCR5 by IFN-γ.

On the basis of our results in the progressive cases in which there was increased chemokine receptor expression, one might predict more CNS inflammation in progressive cases, something we did not observe in our immunohistologic studies. However, we do not know whether activated Th1 type cells that migrate to the CNS undergo rapid apoptosis in the target organ, thus not being visible in the CNS despite their increased expression in the bloodstream. Furthermore, the CNS samples studied were postmortem samples and not from the patients from whom the blood samples were studied. It thus may be that this question can be answered only when T cells can be labeled in the periphery and imaged *in vivo* in the CNS. Further studies are also required to determine whether T cells expressing CXCR3 and/or CCR5 are specific for one of the several myelin autoantigens postulated to play a role in MS, or are specific for an as-yet-undefined antigen.

Chemokine receptor expression has not been studied before in MS, to our knowledge, although previous studies have examined some of the chemokines we analyzed, and the chemokine data assembled thus far are consistent. Studies by McManus *et al.* (18) and Simpson *et al.* (19) detected MCP-1 production by astrocytes, and MIP-1α and MIP-1β production by macrophages, within sections of brains from patients with chronic MS. In addition, MIP-1α has been detected within the cerebrospinal fluid of patients with active disease (20). Last, an anti-MIP-1α antiserum (21) or generation of an immune response to MIP-1α DNA injected as a vaccine (22) prevented CNS infiltration by PBMC in mice with experimental allergic encephalomyelitis, a rodent model of MS.

The studies presented here suggest important roles for MIP-1α, acting via CCR5, and potentially IP-10, acting via CXCR3, in the activation and recruitment of host mononuclear cells to the CNS in MS. In combination with what has been shown in rodent models (23), our data provide a rationale for the use of agents that block CCR5 and/or CXCR3 as a therapeutic approach in the treatment of MS, as well as the use of corresponding mAbs for the peripheral blood monitoring of the clinical course and response to therapy of MS patients. Furthermore, our results suggest that chemokine receptor expression may have relevance for immunologic staging of MS and potentially for other chronic autoimmune or inflammatory processes such as rheumatoid arthritis, autoimmune diabetes, or chronic transplant rejection.

Note Added in Proof. Sorensen *et al.* (24) have recently reported on the expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients.

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Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients

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Chemokines direct tissue invasion by specific leukocyte populations. Thus, chemokines may play a role in multiple sclerosis (MS), an idiopathic disorder in which the central nervous system (CNS) inflammatory reaction is largely restricted to mononuclear phagocytes and T cells. We asked whether specific chemokines were expressed in the CNS during acute demyelinating events by analyzing cerebrospinal fluid (CSF), whose composition reflects the CNS extracellular space. During MS attacks, we found elevated CSF levels of three chemokines that act toward T cells and mononuclear phagocytes: interferon-γ-inducible protein of 10 kDa (IP-10); monokine induced by interferon-γ (Mig); and regulated on activation, normal T-cell expressed and secreted (RANTES). We then investigated whether specific chemokine receptors were expressed by infiltrating cells in demyelinating MS brain lesions and in CSF. CXCR3, an IP-10/Mig receptor, was expressed on lymphocytic cells in virtually every perivascular inflammatory infiltrate in active MS lesions. CCR5, a RANTES receptor, was detected on lymphocytic cells, macrophages, and microglia in actively demyelinating MS brain lesions. Compared with circulating T cells, CSF T cells were significantly enriched for cells expressing CXCR3 or CCR5. Our results imply pathogenic roles for specific chemokine–chemokine receptor interactions in MS and suggest new molecular targets for therapeutic intervention.

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Introduction

Multiple sclerosis (MS), an inflammatory, demyelinating disorder of the human central nervous system (CNS), is the leading cause of nontraumatic neurological disability among young adults in North America (1). Progressive neurological impairment frequently develops during the course of the disease, probably because of irreversible tissue injury (1). Current MS treatments include interferons, corticosteroids, and cytotoxic immunosuppressive agents, often with unsatisfactory outcomes (2). The etiology of the disease remains uncertain but is widely considered to involve organ-specific autoimmune destruction of CNS myelin (3). Innovative therapies for MS seek to abrogate this specific autoreactivity (4).

The histopathology of the MS lesion is characterized by infiltration of the CNS by inflammatory leukocytes, which are considered critical for disease pathogenesis. Inflammatory CNS events during the course of MS are detected *in vivo* by magnetic resonance imaging or by virtue of elevated leukocyte counts in the cerebrospinal fluid (CSF). Recent reports documented a direct correlation between CNS inflammation and clinical progression of MS; these findings focused attention on developing MS treatment strategies to inhibit leukocyte invasion of the CNS (2, 4, 5). For example, novel agents that specifically target membrane glycoproteins associated with leukocyte extravasation are under investigation in MS clinical trials (6, 7).

Investigators seeking to regulate inflammation are guided by extensive research into the molecular mechanisms of leukocyte extravasation (8–10). The blood–brain barrier (BBB), which excludes circulating macromolecules and cells from the CNS, poses an additional consideration for the design of MS therapeutics. Two categories of molecules direct leukocyte migration into inflammatory sites: adhesion molecules and chemoattractants. Among chemoattractants, chemokines (small, proinflammatory chemotactic cytokines) have attracted particular interest because of their potential role in pathogenic inflammation (11–13). Chemokines selectively attract leukocyte subsets; some chemokines act specifically toward neutrophils or eosinophils, others toward monocytes, dendritic cells, or T cells (12). Chemokines appear to act in at least two ways: first, through direct chemoattraction, and second, by activating leukocyte integrins to bind their adhesion receptors on endothelial cells (13, 14). Because

Table 1
CSF chemokine analysis: patient characteristics and CSF formula

Diagnosis	Number	Gender	Age ± SD (years)	Mean CSF WBC: cells/μl ± SD (range)	Abnormal CSF oligoclonal bands (%)	Abnormal CSF/serum albumin ratio[A] (%)
MS[B]	38	21 F/17 M	34 ± 9	13 ± 10 (2-33)	100	2.6
CON[C]	21	12 F/9 M	48 ± 15	2 ± 1 (0-4)	0	0
OND[D]	10	4 F/6 M	59 ± 15	2 ± 1 (1-3)	10	20

[A]Indicates disruption of blood-brain barrier; see Methods for calculations. [B]All had recent symptoms of active demyelinating disease: optic neuritis with or without other symptoms (n = 28); recurrence of MS symptoms (n = 5); isolated brainstem or spinal cord attack (n = 5). [C]Herniated lumbar disc (n = 9) or mechanical low back pain (n = 7); headache with meningitis ruled out (n = 4); brachial plexopathy (n = 1). [D]Cervical spondylotic myelopathy (n = 5); cerebral metastasis (n = 1); carcinomatous meningitis (n = 1); stroke, ALS, axonal polyradiculoneuropathy (n = 1 each). *CSF*, cerebrospinal fluid; *CON*, non-neurological control; *F*, female; *M*, male; *MS*, multiple sclerosis; *OND*, other neurological diseases.

of their number and diversity, chemokines present a bewildering complexity; only recently have specific functions of chemokines in physiology and disease begun to be elucidated. Much of this progress has been made through construction of transgenic and knockout mice, permitting analysis of chemokine action *in vivo* (15). Strikingly, studies using transgenic mice that overexpressed chemokines under control of tissue-specific promoters indicated that chemokines are sufficient to direct migration of target leukocytes into the CNS (16–18).

Several research groups have used experimental autoimmune encephalomyelitis (EAE), a model for MS, to probe CNS chemokine expression and function (19, 20). Karpus and colleagues (21, 22) showed that anti-macrophage inflammatory protein-1α (MIP-1α) antibodies suppressed initial attacks of adoptive-transfer EAE, while anti-monocyte chemotactic protein-1 (MCP-1) antibodies inhibited subsequent relapses, indicating that specific chemokines execute nonredundant functions in this disease model. These findings lent functional significance to prior analyses showing vigorous CNS chemokine expression during EAE, with a remarkably consistent relationship to clinical disease activity (21–30). Importantly, the temporal and spatial patterns of chemokine expression correlated tightly with the distribution of CNS inflammatory infiltrates in EAE (20). Chemokines also influence leukocyte infiltration into the CNS during posttraumatic and ischemic responses (23, 31–36).

Chemokines have been documented in acute and chronic human inflammatory disorders (11). Therefore, results from both experimental models and clinical material motivated consideration of chemokine expression in MS. Examination of MS autopsy material confirmed the distribution of chemokines initially delineated in chronic relapsing murine EAE: MCP-1 was expressed principally by astrocytes near inflammatory infiltrates, while MIP-1α and regulated on activation, normal T-cell expressed and secreted (RANTES) were produced by perivascular inflammatory cells (25, 28, 37–39). MIP-1α was detected at low levels in the CSF of patients with MS and other inflammatory disorders, but not in samples from healthy control subjects (40). CSF levels of other chemokines in MS have not been reported.

In this report, we describe studies of chemokines and chemokine receptors in the CNS in active MS. We studied members of the two major chemokine subgroups: the CXC family, in which two conserved cysteines are separated by a single residue, and the CC chemokines, in which the two cysteines are adjacent. Elevated concentrations of two CXC chemokines, interferon-γ-inducible protein of 10 kDa (IP-10) and monokine induced by interferon-γ (Mig), were detected in the CSF of patients with symptomatic attacks of inflammatory demyelination. CSF levels of the CC chemokine RANTES were also elevated in MS patients. Moreover, $CD4^+$ T cells that expressed the type 3 CXC chemokine receptor (CXCR3), an IP-10/Mig receptor, were enriched in the CSF compared with the systemic circulation. $CD4^+$ and $CD8^+$ T cells in the CSF also expressed type 5 CC chemokine receptor (CCR5), a RANTES receptor, much more frequently than in peripheral blood. Immunohistochemical studies of inflammatory MS brain lesions demonstrated that CXCR3-positive mononuclear cells were an abundant and invariant component of the CNS leukocyte infiltrate. Actively demyelinating MS lesions also contained large numbers of CCR5-positive lymphocytic cells, macrophages, and microglia. CCR1 and CCR3, alternative RANTES receptors, were detected less frequently. Taken in their entirety, these data imply specific roles for IP-10, Mig, and CXCR3, and for RANTES and CCR5, in the MS inflammatory process. Thus, these results suggest new molecular targets for therapeutic intervention in MS.

Methods

Patients. The study was approved by the Scientific Ethics Committee of the Government of Denmark and by the Institutional Review Board of the Cleveland Clinic Foundation. Where mandated, written consent was obtained from all participants.

Two groups of patients were studied. From the first group (n = 69; Table 1), CSF was obtained by lumbar puncture (LP) for chemokine analysis by ELISA. This group included 38 patients that manifested either relapses of established, clinically definite MS or first attacks of demyelinating disease, primarily in the form of optic neuritis with abnormal magnetic resonance imaging (MRI) brain scan and/or abnormal CSF. These patients are designated as MS cases in Table 1. Diagnoses were based on published criteria for clinical research (41–44). No patient received immunomodulatory therapy including corticosteroids before LP; mean time from symptom onset to LP was 14 days. Control subjects (n = 31) in the CSF chemokine ELISA study were divided into two groups (Table 1): controls (CON; n = 21), who had no CNS or major systemic disorder, and other neurological disease controls (OND; n = 10), who had CNS disorders.

The second group of patients underwent LP and venipuncture for analysis of chemokine receptors on cells in CSF and blood. In this group, all patients (n = 9) manifested relapses of established MS. LP and venipuncture were also performed during diagnostic evaluation on patients with aseptic meningitis (n = 5). CSF in excess of that needed for diagnosis was analyzed for chemokine receptors on T cells. For determination of chemokine receptors on circulating cells, venipuncture was performed on healthy volunteers.

CSF processing and routine analysis. CSF was immediately chilled and centrifuged at 250 *g* for 10 min to remove cells. Supernatants were snap-frozen within 20 min of lumbar puncture and maintained at -80°C until analysis.

CSF cell counts were determined with a hemocytometer.

Albumin was measured by rate nephelometry (Array Protein System; Beckman Instruments Inc., Brea, California, USA). Upper limits of normal were four leukocytes per microliter of CSF. For patients under the age of 45 years, normal CSF/serum albumin ratio was 0.0068, while above that age it was 0.0102. CSF/serum albumin ratios were used to evaluate the integrity of the BBB, which normally excludes this serum protein from the CNS. Oligoclonal immunoglobulin bands were detected by isoelectric focusing and immunoblotting (45).

ELISA. Chemokine concentrations were determined by ELISA on coded samples, as described previously (46). Importantly, these assays have been extensively validated on human body fluids from several sites, including the nervous system (46). Chemokine concentrations were measured twice in separate coded ELISA runs on 21 randomly selected CSF specimens, with an interassay variability below 10%.

Brain tissues. Studies were performed on brain tissue taken at autopsy from eight patients with MS and two controls (see Table 3). In the MS material, 14 demyelinated lesions (12 paraffin-embedded tissue samples from six brains, and two fresh-frozen samples from two other brains) were identified by staining for myelin proteins and by hematoxylin–eosin/Luxol fast blue histochemistry. The study was focused on lesions that were actively demyelinating according to established criteria specifying the presence of macrophages containing myelin debris. Furthermore, all lesions were actively inflamed, exhibiting perivascular leukocyte infiltrates (1, 47).

Antibodies. The following antibodies were used for immunohistochemistry: murine monoclonal anti-CXCR3 (LeukoSite Inc., Cambridge, Massachusetts, USA) (48), goat polyclonal anti-CXCR3 (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), murine monoclonal anti-CCR5 (clone 183; R&D Systems Inc., Minneapolis, Minnesota, USA), goat polyclonal anti-CCR5 (Santa Cruz Biotechnology Inc.), rabbit polyclonal anti-CCR5 (kindly provided by W. Marasco, Dana-Farber Cancer Institute, Boston, Massachusetts, USA) (49), murine monoclonal anti-CCR1 (LeukoSite Inc.), goat polyclonal anti-CCR1 (Santa Cruz Biotechnology Inc.), goat polyclonal anti-CCR3 (Santa Cruz Biotechnology Inc.), and murine monoclonal anti-IP-10 (LeukoSite Inc.) (50).

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections were placed on Superfrost Plus slides (Fisher Scientific Co., Pittsburgh, Pennsylvania, USA), deparaffinized in xylenes, and rehydrated through graded ethanol into PBS. Antigen retrieval was performed by microwaving in 1 mM EDTA, pH 8.0 (51). Frozen tissues, sectioned with a cryostat onto Superfrost Plus slides, were dried, stored at –70°C, and fixed in ice-cold acetone immediately before staining.

All immunostaining procedures were performed with the avidin-biotin-horseradish peroxidase complex procedure and 3,3-diaminobenzidine (DAB; Sigma Chemical Co., St. Louis, Missouri, USA) as described previously (1). Slides were treated with 1% H_2O_2 followed by 10% human serum, incubated overnight with primary antibodies at 4°C, washed, incubated with biotinylated secondary antibody at room temperature for 30 min, washed, and incubated with avidin-biotin-horseradish peroxidase complex (Vectastain Elite; Vector Laboratories, Burlingame, California, USA), according to the manufacturer's instructions. After development with DAB substrate, slides were mounted in 80% glycerol in PBS. For each antibody, optimal concentrations of primary and secondary antibody were determined empirically.

Preparation of cells for flow cytometry. Cells were prepared for analysis on a FACS® (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) as described previously (52). Aliquots (12 ml) of CSF were collected directly on ice. After cells were counted in a Jessen Chamber, CSF was centrifuged immediately at 250 *g* for 10 min at 4°C. The supernatant was removed, and cells were washed once in PBS containing 1% BSA and 0.1% sodium azide (PBS/BSA/azide) and resuspended in ice-cold PBS/BSA/azide at a concentration of 40,000 mononuclear cells per milliliter. Blood mononuclear cells were obtained from heparinized venous blood by density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway), washed three times in HBSS with 0.2 % human serum albumin, and resuspended in PBS/BSA/azide at a concentration of 1 million mononuclear cells per milliliter.

To detect CXCR3 on lymphocyte subsets, 100 µl of CSF cells (at least 4,000 mononuclear cells) or blood cell suspensions (100,000 mononuclear cells) were incubated for 30 min with anti-CXCR3 (Clone 49801.111; R&D Systems Inc.) or IgG1 isotype control antibody (Clone 11711.11; R&D Systems Inc.). After one wash and resuspension in FACS/PBS, cells were incubated with FITC-conjugated anti-mouse IgG1 (Clone A85-1; PharMingen, San Diego, California, USA) for 30 min. After a second wash in PBS/BSA/azide, the cells were incubated with peridinin chlorophyll protein (PerCP)-conjugated anti-CD8 (Clone SK1) or allophycocyanin (APC)-conjugated anti-CD4 antibody (Clone SK3) (Becton Dickinson Immunocytometry Systems) for 30 min. Stained cells were then washed twice in PBS/BSA/azide, fixed with 1% paraformaldehyde, and stored at 4°C until analysis. For CCR5 detection on lymphocyte subsets, the following were used: FITC-conjugated anti-CCR5 (Clone 2D7/CCR5; Becton Dickinson Immunocytometry Systems), PerCP-conjugated anti-CD8, and APC-conjugated anti-CD4. The following isotype controls were used: FITC-, phycoerythrin (PE)-, or PerCP-conjugated mouse IgG1 (Clone MOPC-21; Becton Dickinson Immunocytometry Systems), and APC-conjugated anti-CD3 (Clone SK7; Becton Dickinson Immunocytometry Systems). The cells were stained for 30 min in darkness at 4°C, washed twice in PBS/BSA/azide, and fixed in 1% paraformaldehyde before analysis. Samples were analyzed on a four-color FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems). For data analysis, CD4 or CD8 positive lymphocytes were gated, and the marker was set at 1% isotype-control positive events.

Statistics. Differences between group CSF chemokine levels were analyzed with one-way ANOVA, using Bonferroni's *post hoc* test for multiple comparisons; $P < 0.05$ was considered signifi-

Table 2
Chemokine levels (pg/ml ± SEM) in CSF of MS patients and controls

	Non-ELR[A] α-chemokines: T-cell chemoattractants		ELR α-chemokines: neutrophil chemoattractants		β-chemokines: T-cell and monocyte chemoattractants		
Diagnosis	IP-10[B]	Mig	GRO-a	IL-8	RANTES	MCP-1	MIP-1α
MS (n = 38)	936 ± 72	620 ± 32	31 ± 3	50 ± 4	103 ± 16	536 ± 36	ND
CON (n = 21)	319 ± 43	464 ± 36	30 ± 6	29 ± 10	2 ± 2	745 ± 33	ND
OND (n = 10)	586 ± 229	574 ± 106	48 ± 11	36 ± 10	40 ± 26	801 ± 56	ND
MS vs. CON	$P < 0.001$	$P < 0.05$	NS	NS	$P < 0.001$	$P < 0.001$	-
MS vs. OND	NS	NS	NS	NS	NS	$P < 0.001$	-

[A]Glutamate-leucine-arginine motif in the NH_2-terminal domain. [B]ng/ml CSF ± standard error of the mean. *ND*, not detected at assay sensitivity of 10 pg/ml; *NS*, not

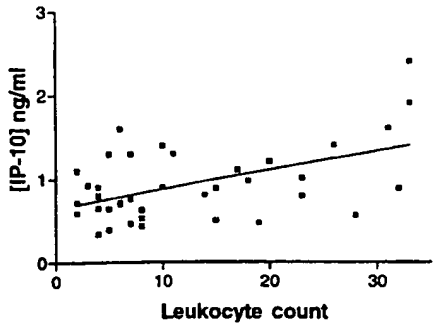

Figure 1
IP-10 concentration correlates with leukocyte count in CSF of MS patients during acute attacks. The scatter diagram shows the relationship between concentration of IP-10 and leukocyte count in the CSF of MS patients (n = 38). Each closed square represents data from one patient. The best-fit line is shown. Pearson r value = 0.4925; two-tailed P = 0.0017. *CSF*, cerebrospinal fluid; *IP-10*, interferon-γ-inducible protein of 10 kDa; *MS*, multiple sclerosis.

cant. The Pearson r value was calculated to analyze correlations between CSF cell counts and chemokine levels. For chemokine receptor expression on T cells, differences between blood and CSF were analyzed by paired t test.

Results

CSF IP-10, Mig, and RANTES levels are elevated in patients with MS attacks. Using enzyme-linked immunosorbent assay (ELISA), we determined CSF levels of seven chemokines – IP-10, Mig, RANTES, MCP-1, MIP-1α, interleukin-8 (IL-8), and growth-regulated oncogene-α (GRO-α) – in MS patients and controls (Table 1). Thirty-eight MS patients were studied during symptomatic episodes of inflammatory demyelination. Of 31 controls, 21 (CON) had noninflammatory benign conditions and were considered free of CNS disease. Ten OND controls had varied CNS disorders, including spondylotic myelopathy, metastasis to the nervous system, or motor neuron disease. MS patients exhibited significantly increased levels of three chemokines (IP-10, Mig, and RANTES), compared with CON (Table 2). One OND control with carcinomatous meningitis had elevated intrathecal levels of multiple chemokines; one OND control with inflammatory polyradiculoneuropathy had very high CSF levels of Mig. Neither of these two OND controls exhibited patterns of CSF chemokine expression characteristic of MS patients.

IP-10 was detected in all MS and control CSF samples, with mean concentration increased nearly threefold in MS compared with CON patients (Table 2). Regression analysis indicated a significant linear relationship between CSF cell count and IP-10 concentration (Fig. 1). We considered whether the differences in IP-10 levels between MS and CON patients could be confounded by the significantly lower age of the MS patients (Table 1; $P < 0.01$). However, there was not a significant relationship between age and CSF IP-10 levels in CON patients (Spearman $\rho = 0.025$; $P = 0.27$). Furthermore, there was a tendency for IP-10 levels to rise with age, suggesting that the differences we observed would be increased by examining age-matched populations. Mean CSF Mig levels were elevated by approximately 50% in MS patients (Table 2). RANTES, undetectable in 20 of 21 CON patients, was identified in the CSF of 58% of patients with MS attacks, with a mean level in the physiologically relevant picomolar range (Table 2). Concentrations of Mig and RANTES did not significantly correlate with CSF cell counts. Levels of MCP-1 were significantly decreased in MS patients, compared with CON or OND. Levels of three other chemokines (MIP-1α, GRO-α, IL-8) were not altered or below the range of detection (Table 2).

Expression of CXCR3 and IP-10 in MS brain lesions. CXCR3 is the IP-10/Mig receptor on activated T cells (53, 54). When sections from inflammatory demyelinating MS brain lesions (Fig. 2, *a* and *b*) (47) were analyzed with anti-CXCR3 antibodies, small, round CXCR3-immunoreactive cells were observed in perivascular leukocyte cuffs (Fig. 3*a*). CXCR3-positive cells, morphologically consistent with lymphocytes, were similar in appearance and distribution to cells detected with the pan-T-cell marker anti-CD3 (Fig. 3*c*). We readily identified CXCR3-positive cells in perivascular cuffs from 14 of 14 lesions present in eight separate MS autopsy brains (Table 3).

To address the frequency with which CXCR3-positive cells were present in MS perivascular infiltrates, all (n = 205) perivascular cuffs in the four largest MS lesions from

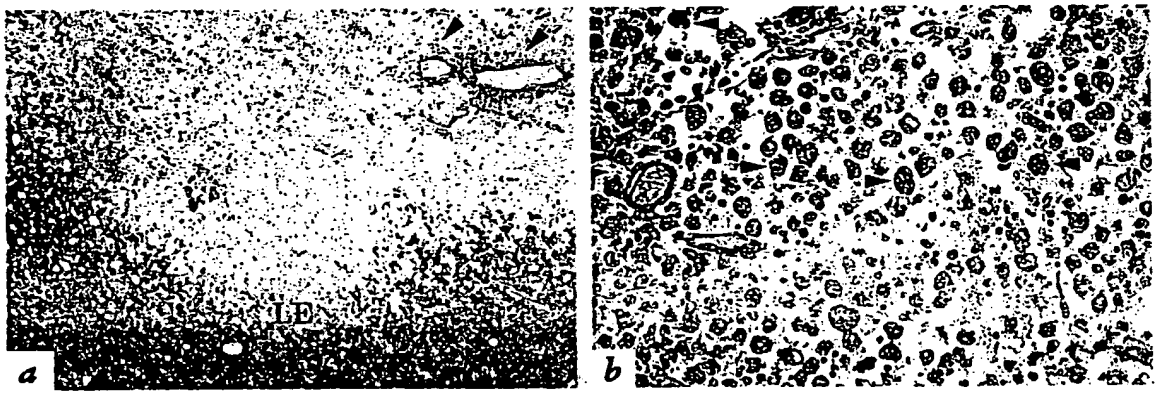

Figure 2
Inflammatory demyelination in an active MS lesion. (*a*) Hematoxylin-eosin histochemistry was combined with Luxol fast blue, which stains myelin blue. This technique reveals a zone of demyelination, abundant perivascular inflammation (*arrowheads*), and an irregular, hypercellular lesion edge (*LE*), below which is normal white matter. ×80. (*b*) Within the lesion, myelin debris, stained blue with Luxol fast blue, is evident within phagocytic macrophages (*arrowheads*), indicating active demyelination. ×170.

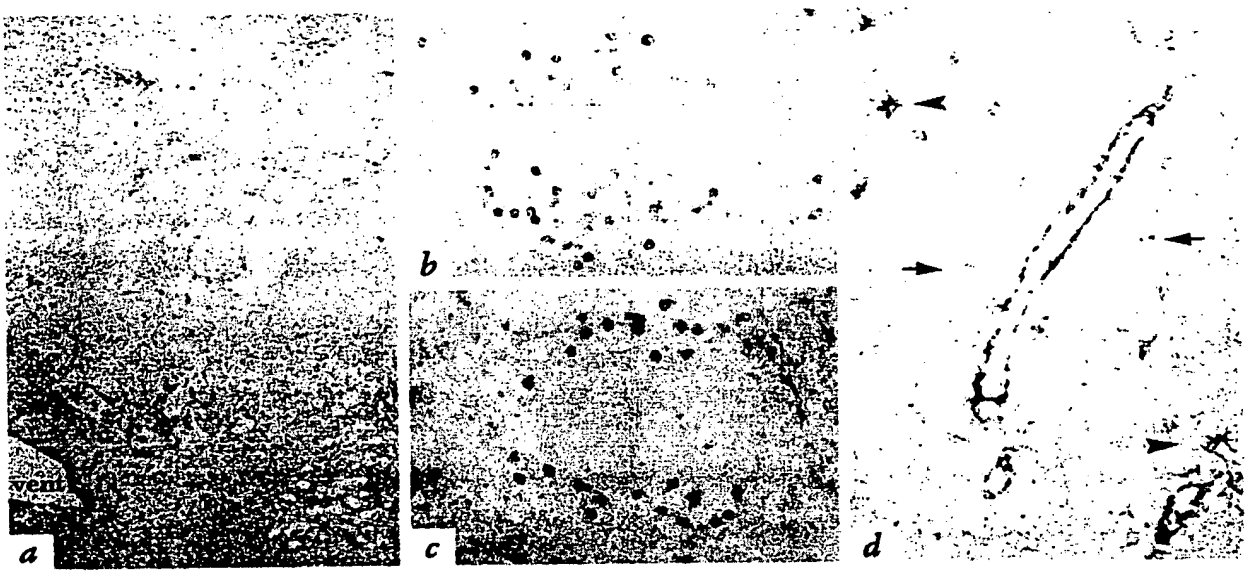

Figure 3
Expression of CXCR3 and IP-10 in MS lesions. (*a*) Numerous CXCR3-immunoreactive cells are present in perivascular inflammatory infiltrates in an acute MS lesion in white matter surrounding a ventricle (*vent*). ×80. (*b*) CXCR3-immunoreactive cells in a perivascular infiltrate exhibit similar morphology and distribution to CD3-positive T cells (*c*). ×170. (*d*) Immunohistochemistry for IP-10 reveals staining of the cell bodies of reactive astrocytes (*arrows*). Intense staining is also seen in processes, consistent with astrocytic end-feet that extend to, and surround, a blood vessel. Scattered reactive astrocytes in the surrounding parenchyma also express IP-10 (*arrowheads*). ×300. *CXCR3*, type 3 CXC chemokine receptor.

three different brains were identified under Nomarski optics and examined for CXCR3-positive cells. Abundant CXCR3-bearing cells were detected in 203 of 205 (99%) perivascular infiltrates, typically comprising 5%–25% of perivascular mononuclear cells. CXCR3-positive cells were not detected in normal-appearing white matter (NAWM) of MS brain or in either of two control brains.

IP-10 immunoreactivity was identified in frozen sections from two autopsy specimens and one biopsy specimen, primarily associated with astrocytes around inflammatory lesions (Fig. 3*d*); the production of IP-10 by astrocytes was described previously in EAE (25, 29, 55). Intense staining was observed in astrocyte foot processes, structures that surround cerebral blood vessels and help maintain BBB integrity (Fig. 3*d*). IP-10 immunoreactivity was not evident in NAWM (not shown).

Distribution of RANTES receptors in MS brain lesions. RANTES, which was significantly elevated in MS CSF specimens (Table 2), engages diverse receptors, including CCR1, CCR3, and CCR5 (13). We examined expression of these receptors in MS lesions.

CCR5 expression is associated with T-helper 1 (Th1) functional differentiation of T cells (53, 56, 57). Using both polyclonal and monoclonal antisera to make distinct portions of the molecule, CCR5 immunoreactivity was demonstrated on cells morphologically consistent with T cells, phagocytic macrophages, and microglia in lesional areas of MS brain tissue (Fig. 4). CCR5-positive cells were identified in actively demyelinating MS lesions (n = 13) in all eight brains.

CCR1, expressed by a majority of circulating T cells and monocytes but not by B cells or neutrophils (58), responds to multiple chemokines, including RANTES. CCR1-positive cells were occasionally detected in the perivascular infiltrates of 5 of 12 acute MS lesions in 5 of 8 autopsied brains (Fig. 5*a*). CCR1-immunoreactive cells exhibited the morphology of small lymphocytes. Their infrequent detection was not due to technical considerations, because all tissues examined contained CCR1-positive cells within blood vessels. CCR3 expression is associated with Th2-polarized T cells (57, 59). CCR3 was detected in only one of the five largest acute MS lesions studied (Fig. 5*b*).

CXCR3-positive CD4⁺ T cells are enriched in CSF compared with peripheral blood. Observations described above implicated IP-10/Mig–CXCR3 signaling in MS-related inflammation. To address the functional significance of these findings *in vivo*, we quantitated CXCR3-bearing T cells in the peripheral blood and CSF of individual MS patients. CXCR3/CD4 double-positive cells in peripheral blood of six MS patients represented 38% of the CD4⁺ population, not significantly different from either neurological controls (not shown) or healthy controls, and consistent with published values (Table 4) (48). In MS CSF, 80% of CD4⁺ cells expressed CXCR3, a significant enrichment compared with peripheral blood (P = 0.004; Table 4). These

Table 3
Chemokine receptor analysis in CNS tissues: patient characteristics

ID number	Age (years)	Gender	Autopsy time (h)	Diagnosis	Number of lesions	Source
35Y94-3N/N1	29	F	17	MS	1	Mayo
W90-37	42	M	18	MS	1	Wistar
YR95-88/5,8[A]	47	M	unk	MS	2	Mayo
2684	51	F	6	MS	1	NNRSB
2765	51	F	9	MS	1	NNRSB
W90-19	60	F	unk	MS	5	Wistar
3Y85-N321; W8	69	F	2	MS	2	Mayo
W90-22	80	F	4	MS	1	Wistar
2607	65	M	26	Non-neurological	–	NNRSB
2601	86	M	13	Non-neurological	–	NNRSB

[A]Brain examined at Mayo (autopsy performed elsewhere). *CNS*, central nervous system; *NNRSB*, National Neurological Research Specimen Bank; *unk*, unknown.

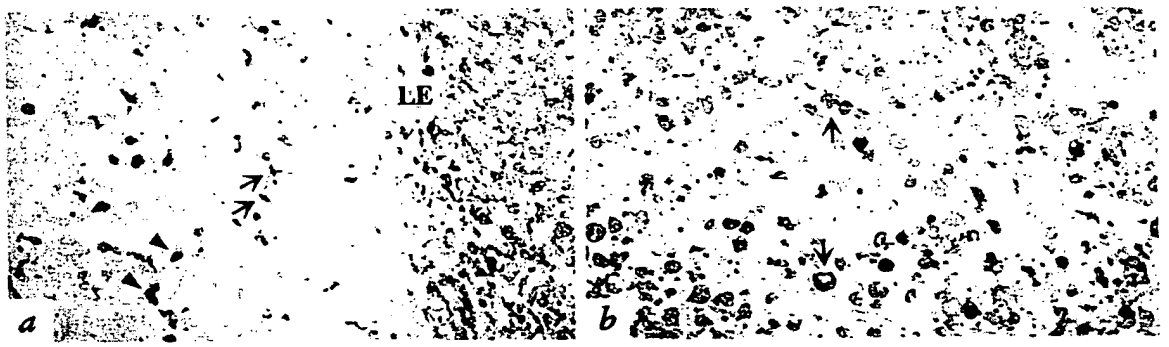

Figure 4
CCR5 expression by multiple leukocyte populations in an active MS lesion. (*a*) Near the lesion edge (*LE*), CCR5 immunohistochemistry reveals scattered elongated process-bearing cells consistent with reactive microglia (*arrows*) and small, round lymphocytic perivascular cells (*arrowheads*), while the lesion (the portion of the panel to the right of the lesion edge) contains numerous CCR5-positive phagocytic macrophages. ×170. (*b*) CCR5-immunoreactive cells in the lesion center are primarily phagocytic macrophages (*arrows*). ×170. *CCR5*, type 5 CC chemokine receptor.

results supported the functional significance of IP-10/Mig–CXCR3 interactions in recruiting CD4+ T cells from the circulation to the intrathecal compartment of MS patients. Eighty percent of circulating CD8+ cells from MS patients were CXCR3-positive, not significantly different from controls. Ninety-seven percent of MS CSF CD8+ cells expressed CXCR3 ($P = 0.044$; Table 4).

Similar findings concerning CXCR3 expression for both CD4+ and CD8+ cells were obtained in analysis of paired blood and CSF samples from five patients with aseptic meningitis (data not shown). This finding was not surprising: meningitis CSF contains T-cell chemotactic activity that is neutralized by anti-IP-10 antibodies. Furthermore, it has been reported that the activated-memory phenotype of CSF T cells in MS and aseptic meningitis is not distinguishable (60, 61).

CCR5-positive T cells are enriched in CSF compared with peripheral blood. To address the functional significance of elevated CSF RANTES and CCR5 expression in MS brain lesions, we compared the percentages of CD4+ and CD8+ T cells that expressed CCR5 in the blood and CSF of seven MS patients. Circulating CD8+ cells in MS patients were 15% CCR5-positive, significantly increased compared with healthy individuals ($P = 0.005$; Table 5). CD8+ cells in MS CSF were further enriched (38%) for CCR5 expression compared with peripheral blood ($P = 0.017$; Table 5). Circulating CD4+ cells in MS patients expressed CCR5 at a low level (4%), with a significant increase to 13% in CSF ($P = 0.018$; Table 5).

Table 4
CXCR3 expression[A] by CD4 and CD8 cells in blood and CSF

		CD4			CD8		
Patient ID	Diagnosis	Blood	Significance	CSF	Blood	Significance	CSF
758	MS	22		87	81		98
759	MS	27		74	76		95
760	MS	44		79	99		99
772	MS	24		72	64		98
773	MS	69		84	97		100
774	MS	44		85	98		99
776	MS	36		73	44		93
Mean ± SEM		38 ± 6	$P = 0.004$[B]	79 ± 2	80 ± 8	$P = 0.044$[B]	97 ± 1
H1	healthy	58		nd	92		nd
H2	healthy	58		nd	84		nd
H3	healthy	49		nd	80		nd
H4	healthy	72		nd	92		nd
H5	healthy	20		nd	77		nd
H6	healthy	27		nd	79		nd
Mean ± SEM		47 ± 8	NS[C]	–	84 ± 3	NS[C]	–

[A]Percent CD4 or CD8 cells that are CXCR3-positive. [B]CXCR3-positive cells in MS CSF compared with MS blood. [C]CXCR3-positive circulating CD4+ or CD8+ cells in control compared with MS subjects. *NS*, not significant; *nd*, not done.

Discussion

To address mechanisms of inflammatory CNS pathogenesis and identify putative molecular targets for therapeutic intervention, we evaluated chemokines and their receptors in MS. In this report, we focus on studies performed on patients with active disease, in the form of either acute symptomatic attacks or inflammatory demyelinating lesions in autopsied brain (47). We found consistent alterations of two ligand-receptor systems: IP-10/Mig–CXCR3 and RANTES–CCR5. These abnormalities included elevated chemokine ligand concentrations in CSF, with corresponding enrichment of receptor-bearing cells in the intrathecal compartment. The distribution and abundance of receptor-bearing cells in affected CNS tissues implied pathogenic significance for the findings of increased CSF chemokine.

CSF chemokine levels are altered during attacks of symptomatic inflammatory demyelination. To initiate studies of chemokine expression in MS, we analyzed CSF, which reflects the composition of the extracellular interstitial fluid of CNS white matter (62). The BBB was largely intact in more than 95% of the MS patients in this study (Table 1); therefore, the elevated CSF chemokine levels most likely reflected intrathecal chemokine production rather than diffusion from blood.

CSF levels of RANTES, Mig, and IP-10 were significantly elevated in MS (Table 2), and there was a positive correlation between the concentration of IP-10 and CSF cell count (Fig. 1). Other chemokines that act toward monocytes and T cells, including MIP-1α, MIP-1β (not shown), and MCP-1, were either undetectable or modestly reduced during MS attacks (Table 2). MIP-1α was previously reported to be slightly elevated in MS subjects, with a mean level of 4 pg/ml (40). Because the lower limit of detection in our ELISA was 10 pg/ml, failure to find measurable CSF MIP-1α was consistent with this prior report. We did not detect the related chemokine MIP-1β in a representative subset of 20 CSF samples (15

MS, 5 CON; data not shown). The neutrophil-directed α-chemokines IL-8 and GRO-α were not altered during the acute MS disease process.

Published data concerning CSF chemokine levels have been obtained primarily from studying infectious disorders. Patients with aseptic/viral meningitis and mononuclear pleocytosis had increased levels of chemokines, such as MCP-1, MIP-1α, and IP-10 (11, 61, 63). In aseptic meningitis, CSF cell counts correlated well with chemokine levels, and chemotactic activity of CSF was abolished by anti-chemokine antibodies (61, 63, 64). Elevated chemokine levels in the CSF of MS patients did not reflect nonspecific inflammatory change in the intrathecal compartment: RANTES was elevated in MS CSF but undetectable in either viral or bacterial meningitis subjects (61, 63, 65). MCP-1 levels were markedly elevated in CSF from meningitis and HIV dementia patients but moderately depressed (Table 2) during episodes of inflammatory demyelination (61, 63, 65, 66).

It was unexpected to observe reduced CSF MCP-1 levels in MS patients undergoing attacks: MCP-1 is expressed by astrocytes bordering MS lesions (38, 39), and robust CNS MCP-1 expression has been repeatedly observed during attacks of EAE. Moreover, anti-MCP-1 antibodies suppressed EAE relapses (22–25). Reduced CSF MCP-1 levels during acute attacks could provide new insight into immunoregulatory characteristics of MS. In particular, it has been proposed that MS relapses are triggered by predominance of Th1, as compared with Th2, lymphokines (67, 68). MCP-1 exposure influences T cells toward Th2 commitment, and MCP-1 expression is regulated by IL-4, the cardinal Th2 cytokine (69–71). We speculate that reduced CSF MCP-1 concentrations corresponded to increased Th1 activity during MS attacks, as reported recently (67). It is also notable that RANTES, IP-10, and Mig, associated with *in vitro* activity toward activated Th1-like T cells, were most significantly elevated during acute MS attacks (57, 72). Our study patients were examined during acute attacks, and it is possible that chemokine levels at other stages of disease may prove to be substantially different.

Our findings in MS patients extend previous observations of CSF chemokines in biologically meaningful (nanomolar) concentrations in controls and patients with CNS disorders (Table 2; and refs. 61, 63–66). Because the composition of the CSF reflects the extracellular interstitial fluid of the white matter, it is most plausible that CNS chemokines originate from cells in the perivascular subarachnoid space and the adjacent parenchyma. Consistent with this concept, we found that IP-10 immunoreactivity was particularly concentrated in astrocyte perivascular foot processes (Fig. 3*d*). Production of chemokines by leukocytes and resident CNS cells near inflamed vessels was described previously in EAE and has recently been reported in MS (25, 28, 37–39).

CNS chemokine receptor expression supports the functional significance of elevated CSF chemokine levels. We found that CXCR3 was the predominant chemokine receptor on lymphocytic cells in both CSF and parenchymal compartments, while CCR5 was present on a minority of lymphocytes in both brain and CSF. These findings support the possibility that activated T cells expressing CXCR3 preferentially gain access to CNS tissues during formation of acute MS lesions. In contrast, in rheuma-

Table 5
CCR5 expression[A] by CD4 and CD8 cells in blood and CSF

		CD4			CD8		
Patient ID	Diagnosis	Blood	Significance	CSF	Blood	Significance	CSF
758	MS	3		11	14		28
759	MS	3		6	17		34
760	MS	4		8	19		44
762	MS	3		6	18		16
767	MS	6		15	13		31
773	MS	7		31	14		72
776	MS	1		15	9		40
Mean ± SEM		4 ± 1	$P = 0.018^B$	13 ± 3	15 ± 1	$P = 0.017^B$	38 ± 7
H1	healthy	2		nd	7		nd
H2	healthy	2		nd	3		nd
H3	healthy	2		nd	11		nd
H4	healthy	1		nd	2		nd
H5	healthy	4		nd	8		nd
H6	healthy	3		nd	9		nd
Mean ± SEM		2 ± 0.4	NS[C]	—	7 ± 1.4	$P = 0.005^C$	—

[A]Percent CD4 or CD8 cells that are CCR5-positive. [B]CCR5-positive cells in MS CSF compared with MS blood. [C]CCR5-positive circulating $CD4^+$ or $CD8^+$ T-cells in control compared with MS subjects.

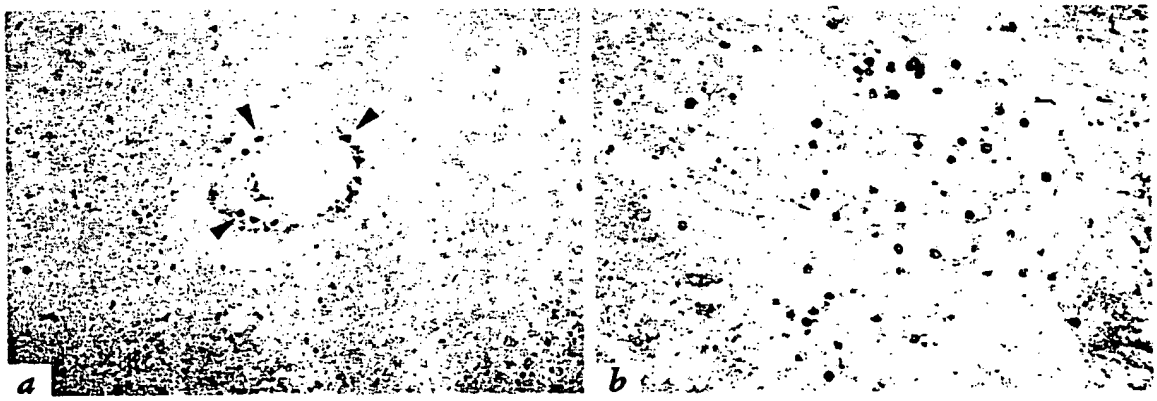

Figure 5
Expression of CCR1 and CCR3 in MS lesions. (*a*) CCR1 immunohistochemistry in an active lesion reveals small, round cells in the perivascular space. Immunoreactive cells are morphologically consistent with infiltrating lymphocytes. ×170. (*b*) Small, round CCR3-immunoreactive cells in a highly active lesion are scattered through perivascular and parenchymal sites. ×170.

toid arthritis, the vast majority (more than 80%) of infiltrating cells in synovial fluid expressed both CCR5 and CXCR3 (48). Although coexpression of chemokine receptors was not addressed in the current studies, such double-positive cells could constitute no more than a minority of the infiltrating population. The relatively modest enrichment of CCR5-positive cells in MS CSF is therefore consistent with the recent observation that individuals homozygous for nonfunctional mutant forms of CCR5 remain susceptible to MS (73).

Given elevated levels of IP-10 and Mig in MS CSF, it was of interest to determine whether cells bearing cognate receptors accumulated in the CSF. By monitoring expression of CXCR3 on T-cell subsets in blood and CSF, we found that 80% of $CD4^+$ cells in MS CSF expressed CXCR3, suggesting that these receptor-bearing cells play a salient role in MS pathogenesis. The functional relevance of elevated CSF IP-10 and Mig levels in MS (Table 2) was further supported by the uniform detection of CXCR3-positive cells in perivascular inflammatory cuffs in MS brain sections. These data are consistent with previous reports showing that anti-IP-10 antibodies depleted the CSF of meningitis patients of chemotactic activity toward T-cell blasts (61). Interestingly, subarachnoid infusion of antisense IP-10 oligodeoxynucleotides reduced EAE disease activity (74).

CCR5, present on a minority of T cells, was highly expressed on phagocytic macrophages and microglia in actively demyelinating lesions (Fig. 4). Given these findings, engagement of CCR5 may be implicated in activating terminal effectors in the lesions of MS (75). CCR5 was expressed on a relatively restricted population of circulating $CD4^+$ and $CD8^+$ cells, with significant enrichment in the CSF. This observation agrees with CSF levels of RANTES, which were significantly elevated over normal concentration, but considerably lower in absolute concentration, than those observed for IP-10 or Mig (Table 2). Although alternative RANTES receptors, such as CCR1 and CCR3, were detected less frequently in the active MS lesions reported here, the significance of these receptors on lesions of different stage or pathogenesis remains quite plausible (76).

CCR5 epitopes have been reported on neurons and astrocytes, as well as on microglia and leukocytes (77). The physiological significance of CCR5 on neurons remains uncertain: neither humans nor mice that lack functional CCR5 appear to be neurologically impaired (78). However, rat hippocampal neurons expressed CCR5 mRNA and demonstrated physiological responses to RANTES *in vitro* (79). In this report, CCR5 was not detected on neurons, using mouse monoclonal antibodies to the NH_2-terminus of CCR5 and goat polyclonal antisera to a COOH-terminal peptide to define CCR5-bearing cells.

In summary, we found consistent expression of specific chemokines and chemokine receptors during active MS. The finding of increased accumulation of CXCR3-positive and CCR5-positive CSF cells imparted functional significance to elevated CSF levels of IP-10, Mig, and RANTES. The pathogenic importance of these data was supported by the uniform detection of CXCR3 and CCR5 on lymphocytic cells, macrophages, and microglia in MS brain lesions. Taken together, these data support the participation of specific chemokines and receptors in the pathogenesis of inflammation and tissue damage in MS and imply that these molecules should be considered as targets for therapeutic intervention.

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Expression of the β-chemokine receptors CCR2, CCR3 and CCR5 in multiple sclerosis central nervous system tissue☆

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Abstract

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) characterised by perivascular inflammatory cell infiltrates and plaques of demyelination. Chemokines have been shown to play an important role in the activation and directional migration of cells to sites of CNS inflammation. The action of chemokines requires the expression of their complementary chemokine receptors by their target cells. We have examined the expression of the β-chemokine receptors CCR2, CCR3 and CCR5 in post-mortem MS CNS tissue using single- and double-labelling immunocytochemistry techniques. Low levels of CCR2, CCR3 and CCR5 were expressed by microglial cells throughout control CNS tissue. In chronic active MS lesions CCR2, CCR3 and CCR5 were associated with foamy macrophages and activated microglia. CCR2 and CCR5 were also present on large numbers of infiltrating lymphocytes. A smaller number of CCR3-positive lymphocytes were present, but we also noted CCR3 and CCR5 on astrocytes in five of the 14 cases of MS investigated, particularly associated with processes around vessels and at the glia limitans. Ligands for CCR2 and CCR3 include MCP-1 and MCP-3 which were co-localised around vessels with the infiltrating leukocytes, but were also present in unaffected areas of cortex. The elevated expression of CCR2, CCR3 and CCR5 in the CNS in MS suggests these β-chemokine receptors and their ligands play a role in the pathogenesis of MS.

Keywords: β-Chemokine receptors; Multiple sclerosis; Astrocytes; Macrophages; Th1 cells

1. Introduction

Chemokines are involved in the pathogenesis of immune-mediated inflammation of the central nervous system (CNS), both in controlling leukocyte migration across brain endothelium and in the activation and movement of cells within the brain parenchyma (Baggiolini, 1998). The biological activities of chemokines are mediated by interactions with their corresponding chemokine receptors (Zlotnik et al., 1999). To date, over 40 chemokines have been described. These small, 6–14-kDa proteins can be classified into four subfamilies depending on the presence and position of a conserved motif of four cysteine residues: CXC (α), CC (β), C (γ) and CX_3C (δ) (Rollins, 1997). Their receptors have been correspondingly named CXCR1-5, CCR1-9, CR and CX_3CR. Within each family, each chemokine receptor can bind several different chemokines and most chemokines bind to more than one receptor (Zlotnik et al., 1999).

The β-chemokine receptors CCR3 and CCR5 have been detected in normal control human CNS tissue associated with microglia (Xia et al., 1998), and are expressed by cultured rat microglia (Boddeke et al., 1999). This is of considerable interest, since CCR3 and CCR5 act as co-receptors for entry of macrophage-tropic strains of human immunodeficiency virus (HIV) into mononuclear phagocytes, including microglia (Choe et al., 1996; Deng et al., 1996; Broder and Collman, 1997; He et al., 1997).

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CCR2 has also been identified on mononuclear phagocytes, and taken together these findings imply that β-chemokines such as RANTES (regulated upon activation, normal T-cell expressed and secreted), monocyte chemoattractant protein (MCP)-1, -2, -3, -4, macrophage inflammatory protein (MIP)-1α and MIP-1β, could all potentially activate and recruit both resident glia and infiltrating haematogenous cells to sites of CNS inflammation.

Investigations have shown that glial cells within the CNS have the capacity to express both chemokines and chemokine receptors during a variety of inflammatory and degenerative conditions (Glabinski et al., 1997; Jiang et al., 1998; McManus et al., 1998a,b; Simpson et al., 1998; Westmoreland et al., 1998; Xia et al., 1998; Klein et al., 1999). For example, increased expression of CCR3 and CCR5 have been detected in the CNS in simian immunodeficiency virus (SIV) infection in macaques (Westmoreland et al., 1998; Klein et al., 1999) and in Alzheimer's disease (Xia et al., 1998), and their ligands in experimental autoimmune encephalomyelitis (EAE) (Glabinski et al., 1997) and multiple sclerosis (MS) (McManus et al., 1998a; Simpson et al., 1998; VanderVoorn et al., 1999). Cytokine-stimulated glial cells have also been shown to express chemokines and chemokine receptors in vitro (Jiang et al., 1998; McManus et al., 1998b).

Chemokines are also thought to be critically important in controlling leukocyte migration across endothelium. This process partly determines the types of immune reaction which develop in different tissues. Recent studies have reported the preferential expression of the chemokine receptors CCR5 and CXCR3 on Th1 cells, and the expression of CCR3 and CCR4 on Th2 cells (Bonecchi et al., 1998). The profile of chemokine receptors on T helper cells may, in part, explain selective recruitment of T cell subpopulations to sites of inflammation, including the predominance of Th1 cells, expressing CCR5 and CXCR3, in MS lesions (Balashov et al., 1999; Simpson et al., 2000). By contrast, CCR2 is not preferentially expressed on either Th subpopulation, although it is upregulated on activated T cells, and is also present on mononuclear phagocytes (Sallusto et al., 1999).

We have previously demonstrated elevated levels of the β-chemokines MIP-1α predominantly associated with glial cells, MIP-1β associated with macrophages/microglia, RANTES associated with perivascular leukocytes, and MCP-1 associated with macrophages and astrocytes in chronic active MS lesions (Simpson et al., 1998); MCP-1 and MCP-3 have also been identified in MS CNS tissue (McManus et al., 1998a; VanderVoorn et al., 1999). As the recruitment of circulating leukocytes and glial cells to sites of inflammation in MS CNS is dependent on chemokine receptor engagement and signalling, we have investigated the expression of the receptors CCR2, CCR3 and CCR5 using immunocytochemistry on post-mortem MS and control CNS tissue.

2. Materials and methods

2.1. Human CNS tissue

Post-mortem CNS tissue sections from 14 MS and 14 age-matched normal controls were provided by the Neuro-Resource Tissue Bank, Institute of Neurology, London, and the Medical Research Council Brain Bank, Institute of Psychiatry, London (as shown in Table 1). CNS tissue blocks, 1 cm^3, were either paraffin-embedded, or snap-frozen in isopentane on liquid nitrogen and stored in airtight containers at −70°C until required for sectioning (as shown in Table 1). Human CNS tissue (10 or 20 μm) was sectioned and collected on Polysine microscope slides (BDH, UK) for immunocytochemical investigation.

The CNS blocks were neuropathologically diagnosed as MS and screened histologically by oil red O (ORO) and Luxol Fast Blue (LFB) staining to show the extent of demyelination and by haematoxylin to assess perivenular inflammatory cuffing. The histological evaluation was scored by two independent observers. Based on these observations the MS tissue was categorised into four groups: (i) normal appearing white matter (NAWM); (ii) acute lesions; (iii) chronic active; and (iv) chronic inactive demyelinated plaques, as defined by Lassmann et al. (1998).

2.2. Single-staining immunocytochemistry

Immunocytochemistry was carried out as previously described (Rezaie et al., 1997; Simpson et al., 1998). The source and dilution of all antibodies used in this study are shown in Table 2. Briefly, paraffin sections were dewaxed and rehydrated, whilst frozen sections were warmed to room temperature (RT). The tissue used in this study was fixed in acetone at 4°C for 10 min, and air-dried for 5–10 min. Sections to be stained using anti-CCR3 and anti-CCR5 antibodies (a kind gift from Dr. John White, SmithKline Beecham, USA) were blocked in goat serum, diluted 1 in 50, for 30 min at RT prior to incubation with a primary rabbit polyclonal antibody directed against either CCR3 or CCR5 for 30 min at RT. Sections to be stained using anti-CCR2 (R&D Systems, UK), and anti-CCR5 (Becton-Dickinson, UK; R&D Systems) antibodies were fixed in methanol/hydrogen peroxide, blocked in normal sera, diluted 1 in 10, for 2 h at RT and incubated with the primary antibody in the absence of lysis buffer for 36 h at 4°C (Rezaie et al., 1997). The optimal antibody dilution of all the antibodies used in this study were selected from a series of antibody titrations, and gave the minimum level of background staining, as shown in Table 2.

The avidin–biotin horseradish peroxidase macromolecular complex method (ABC-HRP) with biotinylated goat anti-rabbit was used as part of the rabbit IgG Vectastain Elite ABC Kit™ (Vector Laboratories, UK) for detection of CCR3 and CCR5 (SmithKline Beecham, USA), whilst

Table 1
Age, sex, death to snap freezing time (DFT), duration of disease (DD), disease course (DC), whether primary progressive (1°), secondary progressive (2°) or relapsing-remitting (RR), whether frozen (F) or paraffin-embedded (P), and cause of death of MS and normal control (NC) CNS tissue used in this study

Tissue	Age (years)	Sex	DD (years)	DC	DFT (h)	Section (μm)	F/P	Cause of death
NC[1a]	49	M	–	–	11	10	F	Myocardial infarction
NC[2a]	80	F	–	–	24	10	F	Pulmonary embolism
NC[3a]	67	M	–	–	38	10	F	Haemorrhage
NC[4a]	28	M	–	–	26	10	F	Cardiac arrest
NC[1b]	68	F	–	–	42	20	F	Ischaemic heart disease
NC[2b]	64	M	–	–	48	20	F	Pulmonary oedema
NC[3b]	75	M	–	–	34	20	F	Myocardial infarction
NC[4b]	70	M	–	–	37	20	F	Peritonitis
NC[5b]	92	F	–	–	27	20	F	Myocardial infarction
NC[6b]	66	M	–	–	67	20	F	Ischaemic heart disease
NC[7b]	69	M	–	–	24	20	F	Congestive heart failure
NC[8b]	77	M	–	–	96	20	F	Myocardial infarction
NC[9b]	63	F	–	–	34	20	F	Myocardial infarction
NC[10b]	58	M	–	–	23	20	F	Myocardial infarction
MS[1a] QS	58	M	14	RR	52	10	F	Unknown
MS[2a] N	60	F	31	RR	12	10	F	Bronchopneumonia
MS[3a] QA	47	F	20	RR	9	10	F	Bronchopneumonia
MS[4a] QS	47	F	7	RR	16	10	F	Cerebrovascular accident
MS[5a] QA & N	59	F	20	RR	13	10	F	Bronchopneumonia
MS[6a] QA	29	F	8	RR	11	10	F	Bronchopneumonia
MS[7a] QC & QS	43	M	18	RR	48	10	F	Bronchopneumonia
MS[8a] N	46	M	11	RR	51	10	F	Bronchopneumonia
MS[9a] QS	60	F	N/K	RR	24	10	F	Bronchopneumonia
MS[1b] QA	47	M	15	N/K	24	20	F	Bronchopneumonia
MS[2b] QA	54	F	N/K	N/K	32	20	F	Pulmonary embolism
MS[3b] QA	32	F	6	N/K	N/K	3	P	Suicide (paracetamol overdose)
MS[4b] QC	56	F	15	N/K	N/K	3	P	Bronchopneumonia
MS[5b] QA	54	F	N/K	N/K	N/K	3	P	Suicide (gunshot)

Tissue obtained from [a]NeuroResource Tissue Bank, Institute of Neurology, London; [b]Medical Research Council Brain Bank, Institute of Psychiatry, London. N, normal appearing white matter; QA, active MS plaque; QS, subacute MS plaque; QC, chronic inactive MS plaque; N/K, not known.

the biotinylated rabbit anti-mouse was used as part of the mouse IgG Dako ABC-HRP Kit™ (Dako, UK) for detection of CCR2 (R&D Systems) and CCR5 (Becton-Dickinson; R&D Systems). The pattern of staining was visualised using 0.2% 3.3'tetrahydrochloride diaminobenzidine (DAB). The sections were counterstained in Meyer's haematoxylin, dehydrated in a graded series of ethanol, cleared in xylene and mounted in DPX (Sigma, UK).

Table 2
Source and specificity of the antibodies used in immunocytochemical investigation of cell marker and chemokine receptor expression in human CNS tissue

Ab Specificity	Isotype	Ig Conc.	Dilution	Source[a]
CD4	$mIgG_1$	50 μg/ml	1:100	Sigma
CD68	$mIgG_2$	430 μg/ml	1:200	Dako
CD68 (PGM-1)	$mIgG_{3K}$	2 ml s/n	1:100	Dako
GFAP	$mIgG_1$	6.5 mg/ml	1:250	Sigma
CCR2	$mIgG_{2B}$	500 μg/ml	1:100–200	R&D
CCR3	rIg[b]	–	1:500	Dr. J White, SKB
CCR5	rIg[b]	–	1:500	Dr. J White, SKB
CCR5	$mIgG_{2A}$	100 μg/ml	1:20–50	Becton-Dickinson
CCR5	$mIgG_{2B}$	500 μg/ml	1:20	R&D
MCP-1	$mIgG_1$	250 μg/ml	1:100	PeproTech
MCP-3	$mIgG_1$	500 μg/ml	1:100	R&D

[a] Sigma Chemical, Poole, Dorset BG17 7BR; Dako, High Wycombe, Bucks HP13 5RE; R&D Systems Europe, Abingdon OX14 3YS; Dr. John White, SmithKline Beecham USA; Becton-Dickinson, Fahrenheit Laboratory Supplies, Rotherham S60 1RR; PeproTech EC, London SW1Y 4JH.
[b] rIgG is a polyclonal antibody. M=mouse.

Sections incubated with blocking serum in the absence of primary antibody were included as a negative control. Mouse isotype-specific antibody controls and normal rabbit IgG were also included to confirm the specificity of the staining pattern.

2.3. Double-staining immunocytochemistry

In order to ascertain which cell types were expressing CCR2, CCR3 and CCR5, double-staining immunocytochemistry was employed. Firstly, the sections were immunostained with the macrophage marker CD68, the astrocyte marker anti-GFAP, or the T-cell marker CD4, using the avidin–biotin peroxidase method as described above. After developing the sections in DAB the slides were immunostained for CCR2, CCR3 or CCR5 expression using the method described above, replacing the rabbit IgG Vectastain Elite ABC-HP Kit™ with the Vector Laboratories avidin and biotinylated alkaline phosphatase macromole-

cule complex (ABC-AP) Vectastain Kit™ (Vector Laboratories). The pattern of CCR expression was visualised using Fast Red (Sigma) or Violet stain (Sigma) as substrate. The sections were then washed in PBS and mounted in PBS/glycerol (1:1).

Negative controls omitting the first and then the second primary antibody, as well as isotype controls were included in all the double-labelling experiments.

Statistical analysis of CCR3 and CCR5 expression by $CD4^+$ T-cells in perivascular inflammatory cell infiltrates in chronic active MS lesions was performed. Double-immunostaining for CD4 and chemokine receptor expression was performed on six post-mortem chronic active MS tissue blocks as described above. The number of double-positive CD4/CCR3 and CD4/CCR5 T-lymphocytes within the perivascular cuffs in these lesions was scored by two independent observers. The results are presented as mean±S.D. Statistical significance was calculated using Student's *t*-test.

3. Results

Post-mortem tissue from 14 normal control and 14 clinically diagnosed MS patients were immunostained to investigate chemokine receptor expression. Immunocytochemistry of both control and MS CNS tissue in the absence of primary antibody, or with appropriate serum or mouse isotype controls, displayed very low levels of background staining on white matter tracts and negative background staining on cortical grey matter (not shown).

Both control and NAWM CNS tissue contained a high proportion of microglia expressing low levels of CCR3 (Fig. 1A) and CCR5 (Fig. 1B) throughout the white matter. The intensities of staining for CCR3 and CCR5 were very similar. In control and NAWM CNS, the majority of CCR3 and CCR5 immunopositive microglia appeared to be in a resting state, as identified by their ramified branched morphology. MS lesions with low levels of inflammation and small amounts of demyelination displayed increased expression of CCR3 and CCR5 associated with amoeboid reactive microglia, with CCR5 also expressed by perivascular inflammatory cells (not shown).

The highest levels of chemokine receptor expression were detected within chronic active MS lesions, correlating with our previous findings that these lesions contain the highest levels of the ligands for these receptors (Simpson et al., 1998). Table 3 gives a summary of chemokine receptor distribution in chronic active MS lesions. The majority of CCR3 and CCR5 immunopositive cells located within the lesion, were large with abundant cytoplasm and were morphologically compatible with macrophages, as confirmed by double staining with the macrophage marker antibody CD68 (Fig. 1C,D). Microglia in the non-demyelinated white matter adjacent to the lesion were immunopositive for CCR3 and CCR5. A proportion of perivascular T-cells expressed CCR3 (Fig. 1E); however, the majority of these $CD4^+$ T-cells were CCR5 positive (Fig. 1F), suggesting infiltrating lymphocytes are of the Th1 phenotype. Statistical analysis of the percentage of CCR3- and CCR5-positive $CD4^+$ T-cells present within the perivascular inflammatory cell infiltrate in chronic active MS lesions showed a statistically significant ($P<0.001$) number of $CD4^+$ T-cells expressing CCR5 (92.8±9.3%) compared to the number of $CD4^+$ T-lymphocytes expressing CCR3 (1.5±1.7%). However, not all of the cells expressing CCR3 or CCR5 could be related to lymphocytes, microglia or macrophages. In addition to the leukocytes, we noted that astrocytes also express surface CCR3 and CCR5 in five of the 14 MS cases investigated. These receptors were weakly expressed on cells at boundaries between grey and white matter (Fig. 2A,B), but were also particularly evident on astrocyte foot processes around vessels in areas of cellular infiltration (Fig. 2C) and beneath the pial surface in areas distant from the lesion (Fig. 2D). Although CCR5 has been detected on simian astrocytes during SIV infection (Westmoreland et al., 1998; Klein et al., 1999), the observation of CCR3 and CCR5 on human astrocytes was unexpected. We therefore confirmed the finding by double-labelling with anti-GFAP. In chronic inactive hypocellular MS lesions GFAP-positive astrocytes predominate over other cell types, but they did not express either CCR3 or CCR5 (not shown). Immunostaining with a number of different commercially available anti-CCR5 antibodies showed a similar pattern of staining.

The receptor CCR2 was identified on infiltrating cells associated with vessels in active plaques. Both mononuclear phagocytes (CD68 positive) and activated lymphocytes within this region were stained (Fig. 3E,F). CCR2 expression was lower on cells distant from vessels, but was still widely distributed in non-affected areas in the cortex on cells which were CD68 negative. Notably, MCP-1 and MCP-3, the principle ligands for CCR2 and CCR3, were also strongly expressed around vessels in the plaques. MCP-3 was confined to the areas around the vessels, while MCP-1 was more widely distributed on both mononuclear phagocytes, infiltrating lymphocytes and astrocytes (Fig. 3A–D).

Table 3
Chemokine receptor distribution in chronic active MS lesions[a]

Chemokine receptor	Plaque			NAWM adjacent to plaque	
	PVC	Mϕ	Astrocyte	Mϕ	Astrocyte
CCR2	++	++	++	−	−
CCR3	+	+++	+	+	++
CCR5	++	+++	+	+	++

[a] −, no staining associated with the cells; +, weak staining; ++, strong staining; +++, all cells stained strongly positive. PVC=perivascular cuff. Mϕ=macrophage microglia

Fig. 1. CCR3 and CCR5 expression in control (Patient NC[4a]) and actively demyelinating MS lesions (Patient MS[4a]), detection using anti-CCR3 and anti-CCR5 antibodies kindly donated by Dr. John White, SKB. Both (A) CCR3 and (B) CCR5 were expressed by resting microglia throughout control CNS white matter (sections were counterstained with haematoxylin). In actively demyelinating MS tissue, expression of (C) CCR3, as indicated by the red stain and (D) CCR5, also indicated by the red stain, was associated with large CD68-positive cells (brown) within the lesion (PL). The arrow indicates an example of a double immunopositive cell. Microglia in the adjacent white matter were very weakly CCR3 and CCR5 positive. Within the plaque infiltrating CD4 T-cells (brown) were (E) predominantly CCR3 (red) negative, but expressed (F) CCR5 (red) (arrow), suggesting infiltrating lymphocytes are of the Th1 phenotype. The CCR3/CCR5-positive CD4-negative cells within the plaque in (E) and (F) were foamy macrophages. The 10-μm thick sections were not counterstained unless otherwise indicated. Blood vessels are indicated by bv. Bars represent 70 μm (A, B, E, F), and 150 μm (C, D).

4. Discussion

Leukocyte migration is essential for immune surveillance of the CNS, and for the recruitment of cells to sites of inflammation. Activated and memory T-cells express higher levels of some adhesion molecules which are required for binding to the inflamed endothelium (Qin et al., 1998), but this does not fully explain the cell-specific recruitment of cells to sites of immune challenge. The selective recruitment of cells is now thought to be directed by chemokines expressed on the lumenal surface of the endothelium, which interact with chemokine receptors

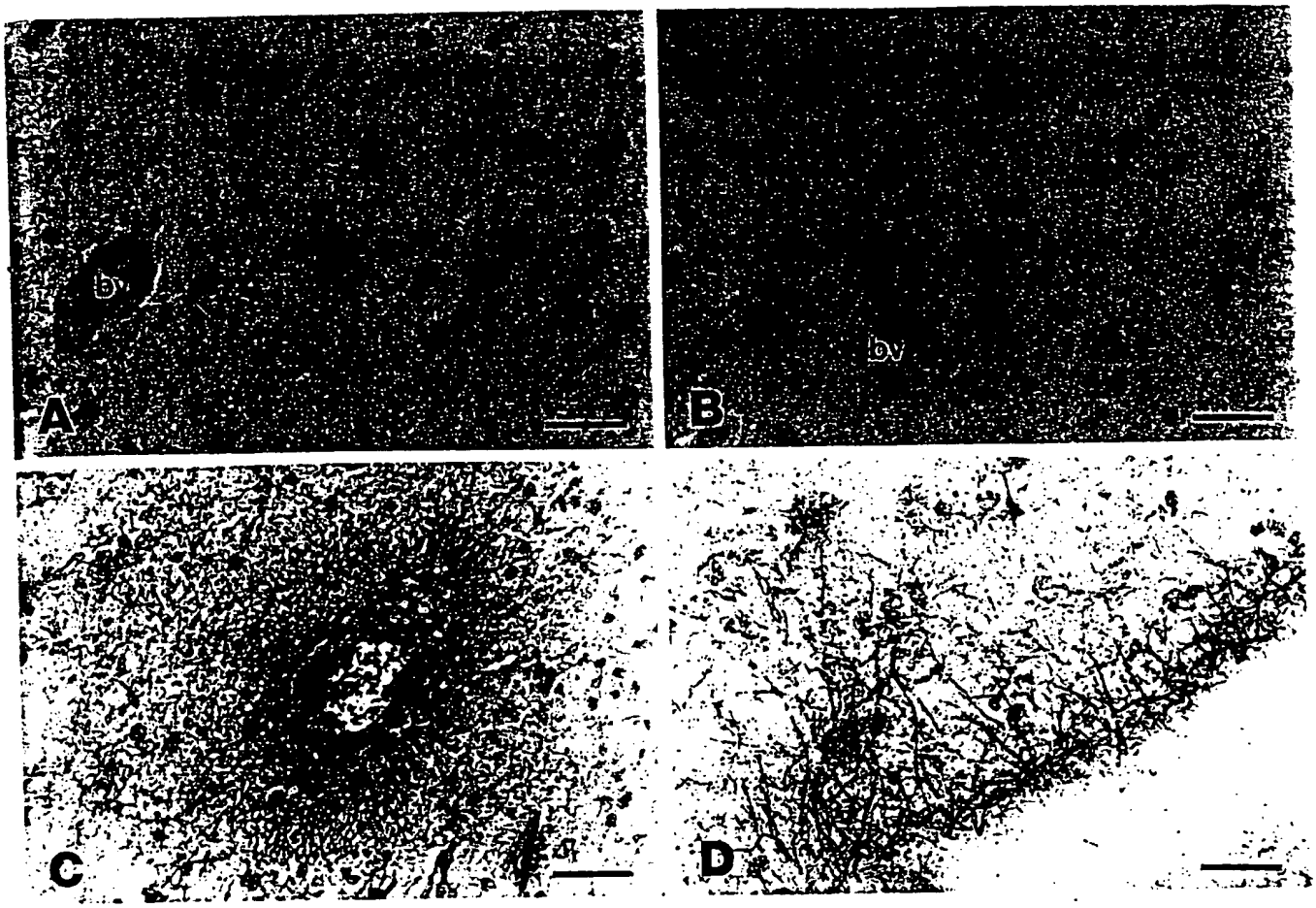

Fig. 2. Astrocyte expression of CCR3 and CCR5 in the frontal cortex of MS (Patient MS[1b]). Astrocytes express CCR3 and CCR5 weakly at the cell surface. Expression is primarily seen at the boundary between grey and white matter (A, B), in perivascular locations (C) and at the pial border (D). (A, B) Stained with rabbit anti-CCR3 (kindly donated by Dr. John White, SKB); (C, D) stained with monoclonal anti-CCR5 (R&D Systems). Blood vessels are indicated by bv. Bars represent 30 μm (A, B, D) and 70 μm (C).

selectively expressed on different populations of leukocytes (Combadiere et al., 1996; Sørensen and Ransohoff, 1998; Sallusto et al., 1999).

In this study, we have investigated CCR2 which is expressed on activated T cells, CCR3 which is selectively expressed on Th2 cells and CCR5 selectively expressed on Th1 cells (Bleul et al., 1997; Sallusto et al., 1998). All three receptors have also been demonstrated on a high proportion of microglia, in both control and NAWM CNS tissue. Following infiltration of inflammatory cells into the CNS, microglia have been shown to transform from a resting to an activated state (Perry et al., 1995). Furthermore, both human and rat microglial cells in vitro have been reported to express CCR3 and CCR5 (He et al., 1997) and to chemotactically migrate along their appropriate β-chemokine ligand gradients (Cross and Woodroofe, 1999). As the extent of perivenular inflammatory cell infiltration increases, the receptor expression by reactive microglia appears to increase. The detection of increased chemokine receptor expression by glial cells is not unique to MS CNS tissue, as the CNS in both macaque monkeys infected with SIV (Westmoreland et al., 1998) and in Alzheimer's disease (Xia et al., 1998) express higher levels of CCR3 and CCR5 associated with some reactive microglial cells, compared to control CNS. Recent studies by Sørensen et al. (1999), and by Balashov et al. (1999) demonstrated that CCR5 was expressed by cells morphologically compatible with T-lymphocytes, phagocytic macrophages and microglia within actively demyelinating MS CNS tissue, supporting the findings reported here. However, of the five lesions investigated by Sørensen, only one contained CCR3-positive cells (Sørensen et al., 1999), compared to all six of the chronic active MS lesions investigated in this study.

In addition to microglia and macrophages, large numbers of other cells in the active plaque borders express the receptors. Morphologically and by double staining the CCR2 and CCR5 positive cells are mostly lymphocytic, and this phenotype is characteristic of an activated Th1 cell phenotype. Previous research has shown that leukocytes in active MS lesions do not express CCR3 or CCR4 (Balashov et al., 1999). In contrast to this report we did identify some CCR3-positive lymphocytes. However it is notable that many of the CCR3-positive, CD68-negative

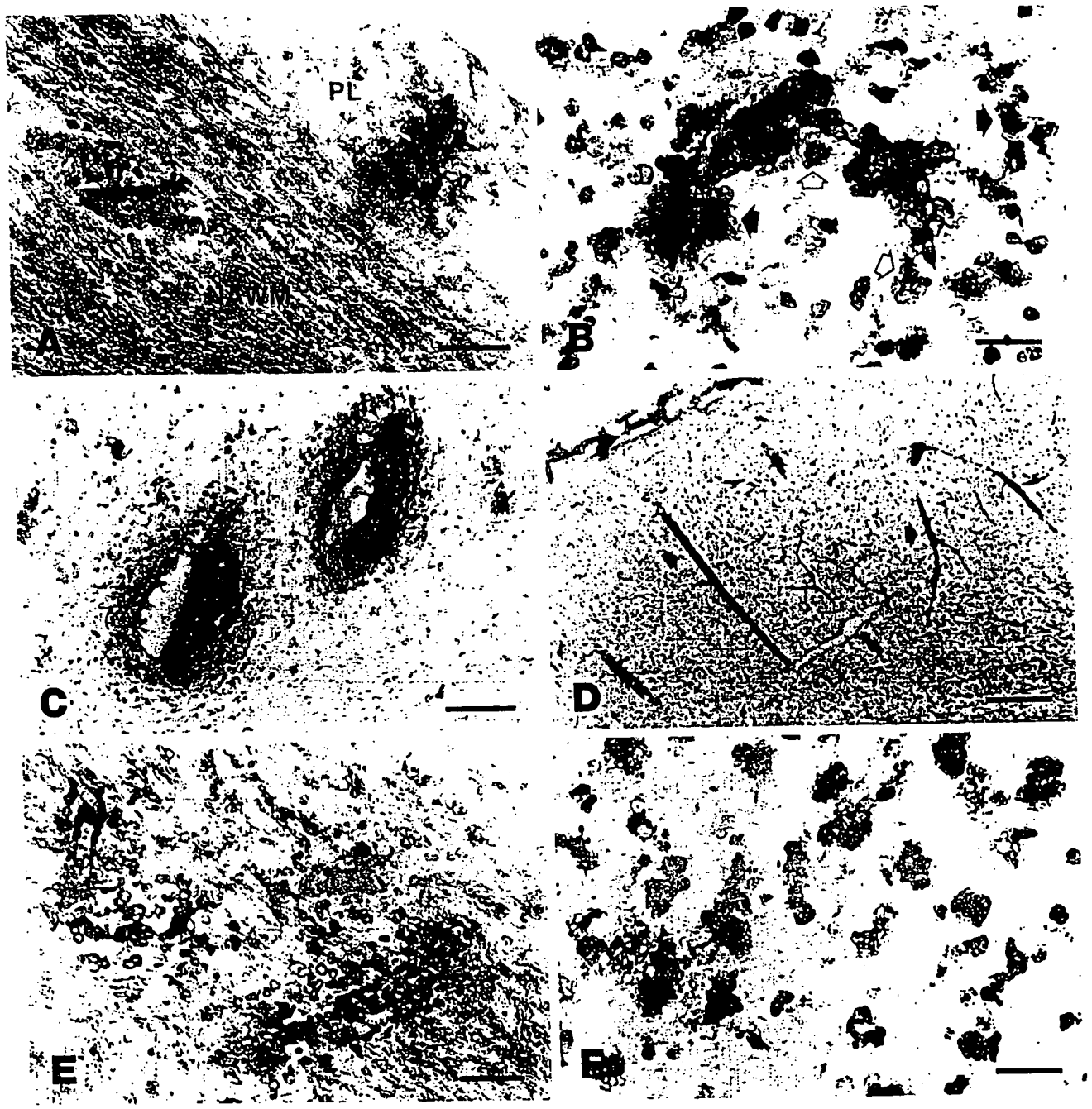

Fig. 3. Double immunolabelling of MCP-1, MCP-3 and CCR2 (Violet stain) and PGM1-positive microglia/macrophages (brown) on frontal cortex of chronic active MS lesion (Patient MS^{1b} and MS^{2b}). The sections have been counterstained with methyl green. MCP-1 is expressed intensely on vessels and associated microglia within the plaque, indicated by PL (A, right of figure) and on the plaque border (A, left of figure) (Patient MS^{2b}). Within the plaque MCP-1 is expressed on both mononuclear phagocytes (solid black arrow) and other PGM-1-negative cells (arrow) (B) (Patient MS^{1b}). MCP-3 is expressed on large vessels within the demyelinating plaque (C) and is strongly associated with vessels in cortical grey matter (D) (Patient MS^{2b}). CCR2 is expressed on perivascular cells within the plaque area (E) (Patient MS^{2b}), including microglia/macrophages (F) (Patient MS^{1b}). Bars represent 150 μm (A, C), 30 μm (B, F), 350 μm (D) and 70 μm (E).

cells (Fig. 1C) are not clearly lymphoid, and we therefore examined these cells in further detail. Some of this staining may be accounted for by weakly staining CCR3-positive astrocytes (Fig. 2A,B), mostly seen at the boundaries of cortical grey and white matter. We show here that CCR5 is also present on astrocytes, and expression is particularly associated with astrocytic foot-processes of the glia limitans at the pial surface and associated with blood vessels. Of the β-chemokine receptors CCR5 has been reported previously on astrocytes in CNS disease (Klein et

al., 1999), but CCR3 has not previously been detected on these cells. The findings indicate that astrocytes located at the blood–brain barrier can potentially respond to β-chemokines, or may act as a sink for the mediators at these sites.

The interactions between chemokines and their receptors is complex (Kunkel, 1999; Zlotnik et al., 1999). In previous reports we have noted the highest level of β-chemokine expression (MCP-1, RANTES, MIP-1α and MIP-1β), in chronic active MS lesions (Simpson et al., 1998). Other studies have identified MCP-1, -2 and -3 in active MS lesions (McManus et al., 1998a). The microglial chemokine receptors demonstrated here respond to different subsets of these chemokines, allowing them to contribute to microglia/macrophage activation in the plaque borders. The role of the chemokines in lymphocyte recruitment is less clear. We have previously reported expression of the α-chemokine receptor CXCR3 on perivascular Th1 cells in chronic active MS lesions (Simpson et al., 2000). The statistically significant presence of CCR5-positive CD4-positive T cells confirms the generally accepted view that MS, like its animal model EAE, is mediated by Th1 cells. The numbers of CCR3-positive lymphocytes were correspondingly significantly lower.

Since CCR2, CCR5 and, to a much lesser extent CCR3, are expressed on the perivascular infiltrating cells, it suggests that β-chemokines could direct transendothelial migration of the leukocytes. It has been debated whether the endothelium itself produces these chemokines, or whether they are released by glial cells and transported across the endothelium to be presented at the lumenal surface. Studies have shown that the CNS vascular endothelial cell lines GP8 and JG2 are capable of producing MCP-1 and RANTES following pro-inflammatory cytokine stimulation (Harkness et al., 1998; Woodroofe et al., 1999; personal observations, PR and DM). Although endothelial cells are capable of producing chemokines, these chemokines are not necessarily present on the lumenal surface.

The question also arises as to why Th2 cells are not equally well recruited to the CNS by their population-selective chemokines, MCP-1, MCP-2 and MCP-3. One possible explanation is that the cellular location of the chemokines is critical. Recent studies have shown that binding sites for MCP-1 and MIP-1α are present on the outer surface of brain microvessels (Andjelkovic et al., 1999). MCP-3, although it is located near blood vessels, is more closely associated with the extracellular matrix (McManus et al., 1998a). Therefore the mere presence of a chemokine does not necessarily mean that it is available to receptors at a site to promote lymphocyte recruitment. To understand the role of chemokines in MS, it is necessary to distinguish their functions in cell recruitment at the blood–brain barrier from their role in cell migration and activation within the brain parenchyma. Leukocyte recruitment and activation are both critical steps in the development of active MS lesions, hence disrupting the binding of chemokines to their receptors may provide a potential therapeutic treatment for MS.

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CCR5 Δ32, matrix metalloproteinase-9 and disease activity in multiple sclerosis

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Abstract

Chemokines and matrix metalloproteinases (MMPs) appear to be crucial in leukocyte recruitment to the central nervous system in multiple sclerosis (MS). CCR5 Δ32, a truncated allele of the CC chemokine receptor CCR5 gene encoding a non-functional receptor, did not confer protection from MS. CCR5 Δ32 was, however, associated with a lower risk of recurrent clinical disease activity. High CSF levels of MMP-9 activity were also associated with recurrent disease activity. These results directly link intrathecal inflammation to disease activity in patients with MS, suggesting that treatments targeting CCR5 or treatment with MMP inhibitors may attenuate disease activity in MS.

Keywords: Cerebrospinal fluid; Chemokine; Matrix metalloproteinase; Multiple sclerosis; CD4 T cells

1. Introduction

CD4 T cells probably orchestrate leukocyte recruitment, leukocyte activation, and ensuing symptoms in multiple sclerosis (MS), an inflammatory demyelinating disease of the central nervous system (CNS) (Karpus and Ransohoff, 1998; Sørensen and Ransohoff, 1998). Leukocyte recruitment is a complex process involving interactions between adhesion molecules, chemotactic factors, and proteolytic enzyme activity (Springer, 1993; Baggiolini, 1998; Luster, 1998). The chemokine family consists of more than 40 small cytokines that exert differential chemotactic activity towards different leukocyte subtypes (Baggiolini, 1998; Luster, 1998). Chemokines are crucially involved in leukocyte recruitment in experimental autoimmune encephalomyelitis (EAE) (Karpus and Kennedy, 1997; Karpus and Ransohoff, 1998, Karpus et al., 1995). Based on conserved amino acid motifs chemokines are divided into four subfamilies of which the CC and the CXC subfamilies include most known chemokines (Baggiolini, 1998; Luster, 1998).

The concentrations of the CC chemokines RANTES (Regulated upon Activation, Normal T cell Expressed and Secreted) and Macrophage Inflammatory Protein (MIP)-1α are increased in cerebrospinal fluid (CSF) from patients with MS (Miyagishi et al., 1995; Sørensen et al., 1999). These chemokines use the CC chemokine receptor CCR5 for signalling (Akhatib et al., 1996; Deng et al., 1996; Dragic et al., 1996; Baggiolini, 1998; Luster, 1998). CCR5 is surface-expressed on T cells secreting proinflammatory (Th1) cytokines, monocytes, macrophages, and microglia, and CCR5 was recently detected on these cell types in active demyelinating MS plaques (Bleul et al., 1997; Rottman et al., 1997; Wu et al., 1997; Bonecchi et al., 1998; Loetscher et al., 1998; Qin et al., 1998; Sallusto et al., 1998; Balashov et al., 1999; Sørensen et al., 1999).

Matrix metalloproteinase (MMP) activity is required for the degradation of basement membrane and extracellular matrix proteins during leukocyte migration (Yong et al., 1998; Cuzner and Opdenakker, 1999). Several MMPs are expressed in inflammatory cells in MS plaques (Cuzner et al., 1996; Anthony et al., 1997; Cossins et al., 1997).

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MMP-9 (gelatinase-B) is, however, the only MMP consistently detected in increased concentrations in CSF from patients with MS (Gijbels et al., 1992; Paemen et al., 1994; Leppert et al., 1998).

Genome screening studies have shown that genetic susceptibility to MS is conferred by multiple genes (Ebers et al., 1996; Sawcer et al., 1996; The Multiple Sclerosis Study Group, 1996; Kuokkanen et al., 1998). In patients of Northern European descent the extended HLA haplotype DRB1*1501–DRB5*0101–DQA1*0102–DQB1*0602 (which includes the DR2, DR15, and Dw2 specificities) remains the only consistently identified susceptibility factor for MS (Compston, 1998). A 32 bp deletion in the CCR5 gene (CCR5 Δ32) is common in healthy Caucasians (Dean et al., 1996; Liu et al., 1996; Samson et al., 1996). CCR5 Δ32 encodes a truncated protein not expressed on the cell surface and CCR5 Δ32 heterozygotes have low surface expression of CCR5 protein (Liu et al., 1996; Wu et al., 1997). Thus, CCR5 Δ32 could influence the inflammatory response in MS as we recently found to be the case in rheumatoid arthritis (Garred et al., 1998). We assessed the role of the CCR5 Δ32 allele in patients with possible onset symptoms of MS (POSMS) or attacks of clinically definite MS (CDMS), and compared CD4 T cell activation (CD25, CD26, CD45 isoform, and HLA-DR expression) and commonly used measures of immune activation in MS (i.e., intrathecal immunoglobulin synthesis, the CSF leukocyte count, MMP-9 activity, and the CSF concentration of neopterin) in patients carrying CCR5 Δ32 and patients carrying normal CCR5 alleles (Hafler et al., 1985; Walker et al., 1985; Ott et al., 1993; Svenningsson et al., 1993; Fredrikson et al., 1987a,b; Sellebjerg et al., 1998a).

2. Materials and methods

2.1. Patients

Sixty-eight patients with POSMS (54 women, 14 men; median age: 30 years, inter-quartile range [IQR]: 26–36 years) and 80 patients with an attack of relapsing-remitting CDMS (54 women, 26 men; median age: 36 years, IQR: 31–41 years) were included in the study (Poser, 1995; Poser et al., 1983). The median Kurtzke Expanded Disability Status Scale (EDSS) score was 3.0 (IQR 2.0–3.0) in patients with POSMS and 3.0 (IQR 2.0–3.5) in patients with CDMS (Kurtzke, 1983). The patients underwent lumbar puncture with a median delay from onset of a demyelinating attack of 15 days in both groups (IQR 9–20 days in POSMS and 9–24 days in CDMS). Intrathecal synthesis of IgG oligoclonal bands was detected in 46 patients (68%) in the POSMS group and in 77 patients (96%) in the CDMS group (Sellebjerg and Christiansen, 1996).

Data from a prospective 1-year follow-up study were available from 26 of the patients with POSMS and 48 with CDMS. These patients were included in two placebo-controlled trials of oral high-dose methylprednisolone (Sellebjerg et al., 1998b, 1999). The follow-up data were considered suitable for the present study because methylprednisolone treatment did not influence the risk of subsequent clinical disease activity. An attack of MS and chronic progressive disease were defined according to established criteria (Poser et al., 1983; Lublin and Reingold, 1996). Data from four of these patients were censored during follow-up as treatment with interferon-β or other disease-modifying drugs was initiated.

Healthy blood donors ($n = 111$) and staff members ($n = 40$) served as a control group for the CCR5 Δ32 allele studies (Garred et al., 1998). Control CSF samples were obtained from 104 patients with noninflammatory diseases (e.g., spinal disk disease, spinal stenosis, low back pain or headache) and, for the flow cytometry studies, also from five patients with inflammatory neurological diseases (encephalitis, cerebral abscess, cerebral vasculitis).

2.2. CCR5 Δ32 and DRB1*1501 genotyping

Genotyping was performed by PCR on genomic DNA isolated from blood cells using the primers (Garred et al., 1998):

5′-CATCATCCTCCTGACAATCGA-3′ (upstream)
5′-CCAGCCCCAAGATGACTATC-3′ (downstream)

The PCR products were analysed by 3% agarose gel electrophoresis. The normal allele was detected as a 157 bp fragment; the CCR5 Δ32 allele was detected as a 125 bp fragment. HLA-DR typing was restricted to detection of the DRB1*1501 allele by a routine method for PCR amplification with sequence specific primers for the PCR reaction (Olerup and Zetterquist, 1992).

2.3. Cerebrospinal fluid analyses

Upper normal limit of the CSF leukocyte count was 4 cells/μl (Andersson et al., 1994). CSF and serum concentrations of albumin, IgA, IgG, IgM, and myelin basic protein (MBP; age-dependent reference limits (van Engelen et al., 1992)) were analysed by various immunoassays. The CSF-serum albumin concentration quotient (Q_{alb}, age-dependent reference limits (Blennow et al., 1993) and intrathecal synthesis of IgA, IgG, and IgM (upper reference limits 0 mg/l, (Sellebjerg et al., 1998a)) were calculated as previously described. Neopterin was measured by a radioimmunoassay with a measuring range of 1.35–111 nM (Neopterin ELISA, IBL, Hamburg). Matrix metalloproteinase-9 (MMP-9) was measured as 90 kDa gelatinase activity by zymography in polyacrylamide gels co-polymerized with 0.1% gelatine (Gijbels et al., 1992). A fetal calf serum preparation defined to contain 1000 arbitrary units (AU) of MMP-9 activity was used as standard. The measuring range of the MMP-9 assay was 0.4–50 AU.

Table 1
Carrier frequency and homozygosity frequency for the CCR5 Δ32 allele

	Carrier frequency	Homozygosity
Possible onset symptom of multiple sclerosis	15/68 (22%)	1/68 (1.5%)
Clinically definite multiple sclerosis	17/80 (21%)	0/68 (0%)
DRB1*1501 negative patients	16/76 (21%)	0/76 (0%)
DRB1*1501 positive patients	16/72 (22%)	1/72 (1%)
Healthy control subjects	39/151 (26%)	2/151 (1.3%)

2.4. Flow cytometry

The following fluorochrome-conjugated antibodies were used in the study: anti-CCR5 (clone 2D7, PharMingen, San Diego, CA), anti-CD3 (clone SK7, Becton Dickinson Immunocytometry Systems, San Jose, CA), anti-CD4 (clone SK3, Becton Dickinson), anti-CD8 (clone SK1, Becton Dickinson), anti-CD14 (clone MϕP9, Becton Dickinson), anti-CD16 (clone NKP15, Becton Dickinson), anti-CD19 (clone SJ25C1, Becton Dickinson), anti-CD25 (clone B-B10, Serotec, Oxford, UK), anti-CD26 (clone 4EL-1C7, Coulter Immunology, Hialeah, FL), anti-CD45R0 (clone UCHL1, Serotec), anti-CD45RA (clone B-C15, Serotec), anti-HLA-DR (clone B-F1, Serotec), IgG1 isotype controls (Becton Dickinson). Mononuclear cells from peripheral blood (isolated by gradient centrifugation on Lymphoprep™, Nycomed, Oslo, Norway) and CSF were stained with monoclonal antibodies, washed, and stored fixed in 1% paraformaldehyde at 4°C until analysis on a three-color FACScan™ flow cytometer (Becton Dickinson) (Sellebjerg et al., 1998c); a four-color FACS Calibur™ flow cytometer (Becton Dickinson) was, however, employed for the CCR5 expression studies.

2.5. Magnetic resonance imaging

MRI of the brain and the brain stem was carried out on a 1.5 T Siemens Magnetom (Frederiksen et al., 1996). Two different pulse sequences were used: a T1-weighted sequence with a repetition time (TR) of 520 ms and an echo time (TE) of 15 ms, and a T2-weighted sequence with two spin echoes (TR = 2500 ms, TE = 15 and 90 ms). The T1-weighted pulse sequence was repeated approximately 10 min after intravenous injection of gadolinium (Gd)-DTPA (0.1 mmol/kg body weight). The presence of lesions enhancing after administration of Gd-DTPA on T1-weighted images, and the presence of lesions highly suggestive of MS on T2-weighted images (Paty grade 1a or 1b) was noticed (Paty et al., 1988).

2.6. Statistics

Categorical data were analysed by Fisher's exact test. Normal distributed variables were analysed by the *F*-test, ANOVA, and the *t*-test. Nonparametric methods (Kruskal–Wallis, Mann–Whitney *U*-test, and Wilcoxon tests) were used for variables that were not normal distributed. The risk of new disease activity was initially analysed by Kaplan–Meier plots and the log-rank test. Odds ratios (with 95% confidence intervals, CI) for new disease activity risk were estimated by Cox regression analysis. A significance level of 0.05 was employed; a Bonferroni correction was employed in post hoc tests.

3. Results

3.1. CCR5 Δ32 in patients and control subjects

Carrier rate and homozygosity frequency for the CCR5 Δ32 allele was comparable in patients with POSMS, CDMS, and control subjects (Table 1). The CCR5 Δ32 allele was also detected in a comparable percentage of DRB1*1501 positive and DRB1*1501 negative patients (Table 1). The age of onset of disease was somewhat lower in patients carrying CCR5 Δ32 (median 27 years) than in the remaining patients (median 30 years) but the difference was statistically significant only when the analysis was restricted to patients with intrathecal synthesis of IgG oligoclonal bands ($n = 123$; median age of onset 25 and 30 years, respectively; Mann–Whitney, $p = 0.03$). Age, sex, EDSS score, and disease duration did not differ in patients carrying CCR5 Δ32 and patients carrying normal CCR5 alleles.

3.2. CCR5 Δ32 and T cell subsets

Consistent with a role of CCR5 in leukocyte recruitment more CD4 T cells, CD8 T cells, and monocytes in

Table 2
Flow cytometry measurements of the percentage of mononuclear cell types in peripheral blood and CSF expressing CCR5 in six patients with possible or clinically definite multiple sclerosis (median, range; statistical testing by the Wilcoxon test)

	Peripheral blood	Cerebrospinal fluid	*p*-value
CD4 T cells	4%, 0.1–13%	17%, 0.1–31%	$p = 0.03$
CD8 T cells	11%, 0.3–22%	30%, 0.8–59%	$p = 0.03$
Monocytes (CD14+)	4%, 0–61%	76%, 27–100%	$p = 0.03$
NK cells (CD14−, CD16+)	2%, 0.4–5%	4%, 0–33%	NS
B cells (CD19+)	4%, 2–13%	13%, 0–23%	NS

CSF than in peripheral blood expressed CCR5. In contrast, CCR5 played no obvious role in B cell or NK cell recruitment (Table 2). The percentage of CD4 T cells in peripheral blood expressing CD26 was significantly higher in patients carrying the CCR5 Δ32 allele ($p < 0.001$) and in patients with normal CCR5 alleles ($p < 0.001$) than in control subjects (Fig. 1). The percentage of CD4 T cells in CSF expressing CD26 was not higher in patients with MS (Fig. 1), but the percentage was less variable in patients carrying CCR5 Δ32 than in patients not carrying this allele ($F = 5.3$; $p = 0.009$) or control subjects ($F = 9.1$, $p = 0.001$). Blood and CSF CD4 T cell expression of CD25, CD45 isoform expression, and HLA-DR was not associated with presence of the CCR5 Δ32 allele.

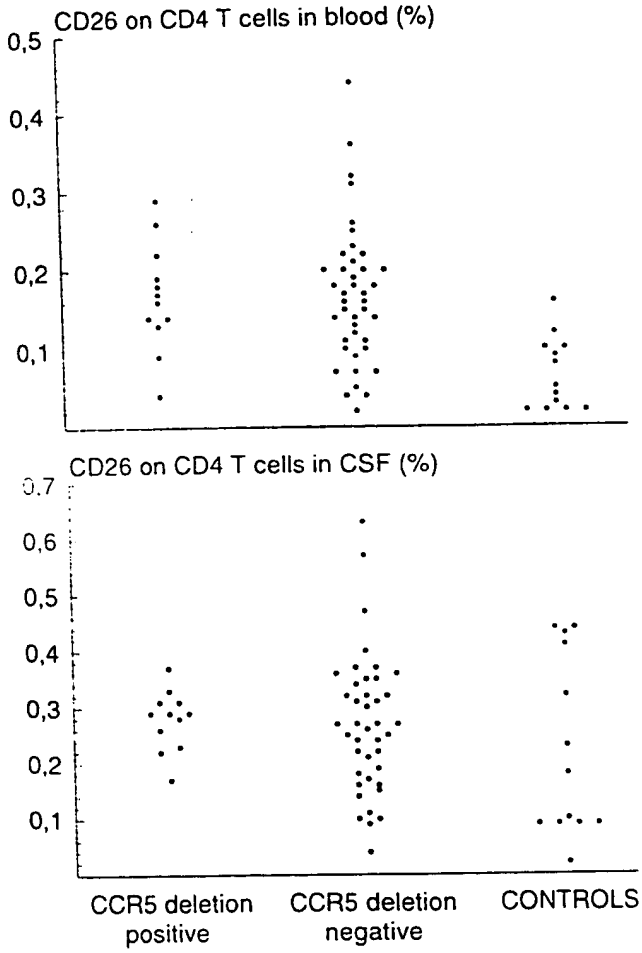

Fig. 1. CD4 T cell expression of CD26 in 53 patients with possible onset symptoms or clinically definite multiple sclerosis (12 carrying the CCR5 Δ32 allele) and 13 neurological control subjects. The percentage of peripheral blood CD4 T cells expressing CD26 differed significantly in the three groups (upper panel; ANOVA, $p < 0.001$) and was higher in both patient subgroups than in the control subjects (*t*-test, both $p < 0.001$). The percentage of cerebrospinal fluid (CSF) CD4 T cells expressing CD26 was less variable in patients carrying the CCR5 Δ32 allele than in the two other patient groups (*F*-test, both $p < 0.01$), but the mean percentage of CD4 T cells in CSF expressing CD26 did not differ significantly in the three groups (lower panel).

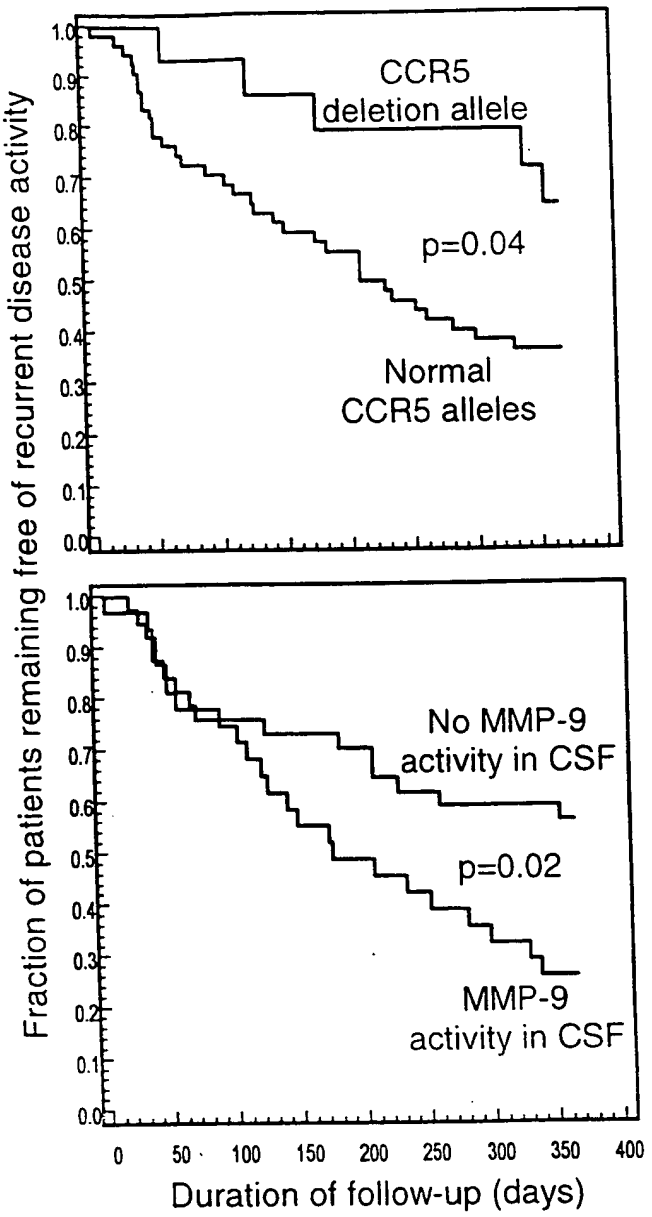

Fig. 2. Kaplan–Meier plot of the risk of new disease activity (attack or onset of a chronic progressive disease course) in 15 patients carrying the CCR5 Δ32 allele and 55 patients with normal CCR5 alleles (upper panel); and in 32 patients with matrix metalloproteinase (MMP)-9 activity in cerebrospinal fluid (CSF) and 38 patients without MMP-9 activity in CSF (lower panel). Statistical analysis was by the log-rank test..

3.3. MMP-9 and other measures of intrathecal inflammation

In the 148 patients with POSMS or CDMS an increased CSF leukocyte count was observed in 57%; an increased CSF concentration of MBP was observed 66%; an increased Q_{alb} was observed in 10%; increased intrathecal immunoglobulin synthesis rates was observed in 12% for IgA, 62% for IgG, and 16% for IgM (16%). These variables did not differ in patients carrying CCR5 Δ32 and the remaining patients. MMP-9 activity was detected in CSF

from 35 out of 74 patients with MS or POSMS studied (47%) whereas MMP-9 activity was present in CSF from only three of 32 neurological control subjects (9%, $p < 0.001$). The median CSF concentration of neopterin was 3.4 nM (IQR 2.7–4.7 nM) in the 74 patients with POSMS or CDMS and 2.8 nM in 39 neurological control subjects (IQR 1.6–3.6 nM, $p = 0.02$). None of these CSF measures of disease activity differed in patients carrying CCR5 Δ32 and patients carrying normal CCR5 alleles.

3.4. CCR5 Δ32, MMP-9, and clinical disease activity

The risk of recurrent clinical disease activity was assessed in 70 patients who were followed up prospectively until the next exacerbation or for approximately 1 year after a demyelinating attack. Within the follow-up period a new attack occurred in 10 out of 26 patients with POSMS (38%) and 25 out of 44 patients with CDMS (57%), and onset of chronic progression occurred in four patients with CDMS (9%). The patients with CDMS had a higher risk of recurrent disease activity (a new attack or onset of chronic progression) than did the POSMS patients ($p = 0.003$). The 15 carriers of CCR5 Δ32 had a lower risk of recurrent disease activity than did the 55 patients with normal CCR5 alleles, and onset of chronic progression was only observed in patients with normal CCR5 alleles (Fig. 2). Patients with MMP-9 activity in CSF also had an increased risk of recurrent disease activity (Fig. 2). In a multivariate Cox regression analysis the odds ratio of recurrent disease activity were: 4.6 (95% CI 2.1–10, $p = 0.0001$) in patients with CDMS compared to patients with POSMS; 0.32 (95% CI 0.12–0.83, $p = 0.02$) in patients carrying the CCR5 Δ32 allele compared to patients with normal CCR5 alleles; and 2.8 (95% CI 1.4–5.5, $p = 0.003$) in patients with MMP-9 activity in CSF compared to patients without. Two almost identical multivariate Cox regression models were obtained when the four patients with incomplete follow-up were included in the analysis, and when the four patients who had onset of chronic progression during the follow-up period were excluded from the analysis.

The influence of a series of other factors on the risk of recurrent disease activity and their distribution according to CCR5 genotypes and presence of MMP-9 activity in CSF was also assessed (Table 3). The risk of recurrent disease activity was higher in patients with Kurtzke EDSS scores above 3.0, above median levels of intrathecal IgG synthesis, or above median concentrations of MBP in CSF. No significant difference in the distribution of potential confounding factors were identified in patients carrying CCR5 Δ32 and patients with normal CCR5 alleles. In contrast a higher percentage of patients with MMP-9 activity in CSF had intrathecal synthesis of IgG oligoclonal bands, enhancing lesions on MRI, above median levels of intrathecal IgG synthesis, CSF leukocyte counts, CSF concentrations of neopterin and CSF concentrations of MBP, and lower than median age. None of these factors or any of

Table 3
Odds ratios (with 95% confidence intervals) of recurrent disease activity in 70 patients with possible onset symptoms or clinically definite multiple sclerosis (MS) as assessed by univariate Cox regression analysis
For quantitative variables the patients were dichotomized according to median values. The distribution of the factors assessed was also compared in: patients carrying CCR5 Δ32 ($n = 15$) and normal CCR5 alleles ($n = 55$); and in patients with matrix metalloproteinase (MMP)-9 activity ($n = 32$) or without MMP-9 ($n = 38$) activity in CSF (statistical testing by Fisher's exact test: NS = not significant)

	Odds ratio of recurrent disease activity	CCR5 Δ32 allele		MMP-9 activity in CSF	
		Absent	Present	Absent	Present
CCR5 Δ32 allele carrier	0.4 (0.2–1.0), $p = 0.05$	–	–	24%	19% (NS)
CSF-serum albumin quotient ≥ 0.0047	0.6 (0.3–1.1), $p = 0.1$	49%	67% (NS)	53%	53% (NS)
Age ≥ 33 years	0.8 (0.4–1.5), $p = 0.4$	51%	47% (NS)	66%	31% (NS)
Intrathecal IgA synthesis ≥ −1.47 mg/l	1.1 (0.6–2.1), $p = 0.7$	51%	47% (NS)	55%	44% (NS)
CSF concentration of neopterin ≥ 3.4 nM	1.1 (0.6–2.0), $p = 0.9$	55%	33% (NS)	32%	72% ($p = 0.002$)
Methylprednisolone treatment	1.1 (0.6–2.0), $p = 0.9$	53%	53% (NS)	47%	59% (NS)
Men	1.2 (0.6–2.3), $p = 0.6$	66%	53% (NS)	58%	69% (NS)
DRB1* 1501 allele carrier	1.2 (0.6–2.2), $p = 0.6$	46%	53% (NS)	34%	63% ($p = 0.03$)
Intrathecal IgM synthesis ≥ −0.55 mg/l	1.2 (0.6–2.3), $p = 0.6$	53%	47% (NS)	47%	56% (NS)
CSF leukocyte count ≥ 7/μl	1.6 (0.8–3.0), $p = 0.2$	51%	53% (NS)	26%	81% ($p < 0.001$)
IgG oligoclonal bands in CSF	1.7 (0.5–5.5), $p = 0.4$	89%	93% (NS)	82%	100% ($p = 0.01$)
Duration of symptoms ≥ 12 days	1.8 (0.9–3.4), $p = 0.08$	51%	47% (NS)	42%	59% (NS)
CSF concentration of MBP ≥ 1.95 μg/l	1.9 (1.0–3.5), $p = 0.05$	46%	53% (NS)	34%	63% ($p = 0.03$)
T2-weighted MRI highly suggestive of MS	1.9 (0.8–4.1), $p = 0.1$	71% ($n = 52$)	71% ($n = 14$; NS)	65% ($n = 37$)	79% ($n = 29$ NS)
Intrathecal IgG synthesis ≥ 14.6 mg/l	2.0 (1.0–3.8), $p = 0.04$	53%	33% (NS)	16%	88% ($p < 0.001$)
Abnormal Gadolinium-enhanced MRI	2.0 (0.8–4.9), $p = 0.1$	73% ($n = 44$)	70% ($n = 10$; NS)	58% ($n = 31$)	91% ($n = 23$; $p = 0.01$)
Kurtzke EDSS score ≥ 3.0	2.1 (1.1–3.9), $p = 0.02$	31%	33% (NS)	34%	28% (NS)
MMP-9 activity in CSF	2.1 (1.1–3.9), $p = 0.03$	47%	40% (NS)	–	–
Clinically definite MS	2.8 (1.4–5.8), $p = 0.005$	60%	73% (NS)	68%	56% (NS)

the other factors assessed were, however, statistically significant when introduced in multivariate Cox model by neither forward nor backward stepwise regression analyses.

4. Discussion

The CCR5 gene is located at chromosome 3p21, an area to which definite linkage to MS was not detected in genome screening studies (Ebers et al., 1996; Sawcer et al., 1996; The Multiple Sclerosis Study Group, 1996; Kuokkanen et al., 1998). No association between the CCR5 Δ32 allele and susceptibility to MS and no evidence of an association between the CCR5 Δ32 and DRB1*1501 alleles was found in neither a previous nor the present study (Bennetts et al., 1997). Two recent studies have, however, suggested that genes in the vicinity of the human CC chemokine cluster on chromosome 17q may influence susceptibility to MS, and in both studies an interaction with the MS-associated DRB1*1501 allele was suggested (Naruse et al., 1996; Coraddu et al., 1998; Fiten et al., 1999).

CCR5 gene knock-out mice have deficient macrophage function, less efficient clearing of Listeria infections, and less pronounced responses to bacterial lipopolysaccharides. In contrast, delayed-type hypersensitivity and antibody production are enhanced, presumably reflecting compensatory mechanisms (Zhou et al., 1998). Humans carrying CCR5 Δ32 have no overt immunodeficiency, but this allele influences host susceptibility and prognosis in HIV infection (Dean et al., 1996; Liu et al., 1996; Samson et al., 1996; Eugen-Olsen et al., 1997). Furthermore, lower disease activity is seen in rheumatoid arthritis patients carrying CCR5 Δ32. These patients have lower rheumatoid factor titres and less morning stiffness than do patients carrying normal CCR5 alleles (Garred et al., 1998). We also found a lower risk of recurrent disease activity in CCR5 Δ32 positive patients with POSMS or CDMS, suggesting that signalling through CCR5 may be involved in the pathogenesis of both rheumatoid arthritis and MS. The patients included in the present study were well suited for an analysis of the risk of recurrent disease activity because all underwent lumbar puncture within one month from the onset of a demyelinating attack, but the low number of patients developing chronic progressive disease during follow-up precluded an assessment of the risk of progression in patients carrying CCR5 Δ32. Additional follow-up studies of larger patient cohorts or magnetic resonance imaging studies are therefore needed before the full pathogenetic role of CCR5 Δ32 and other polymorphisms in the CCR5 gene can be assessed (McDermott et al., 1998).

The chemokines MIP-1α, MIP-1β, and RANTES utilize CCR5 for signaling (Akhatib et al., 1996; Deng et al., 1996; Dragic et al., 1996; Baggiolini, 1998; Luster, 1998). MIP-1β immunoreactive cells have been detected in MS plaques but not in CSF from patients with MS (Simpson et al., 1998; Sørensen et al., 1999). MIP-1α immunoreacive cells can be detected in MS plaques and MIP-1α is present in low levels in CSF from patients with MS (Miyagishi et al., 1995; Balashov et al., 1999). RANTES concentrations are, however, substantially higher, suggesting that RANTES may be a are more important CCR5 ligand in MS CSF (Sørensen et al., 1999). Of the known RANTES receptors CCR1, CCR3 and CCR5, CCR5 can be detected in the majority of MS plaques where it is expressed on macrophages, microglia, and a subset of perivascular lymphocytes. CCR1 is less abundantly expressed than CCR5, and CCR3 is rarely expressed in MS plaques (Balashov et al., 1999; Sørensen et al., 1999).

The results of the present study give only an indirect indication of the mechanisms that may underlie the effect of CCR5 Δ32 in MS. A panel of routine CSF measures of immune activation and disease activity did not differ in patients carrying CCR5 Δ32 and patients carrying normal CCR5 alleles but the absence of an effect of CCR5 Δ32 on intrathecal immune activation during an established MS attack does not rule out an effect of this allele during the very early stages of an attack. CD4 T cells are believed to orchestrate the intrathecal inflammation in patients with MS (Sørensen and Ransohoff, 1998). In agreement with the findings in the CCR5 knock-out mouse patients carrying CCR5 Δ32 had no overt defect in CD4 T cell recruitment (Zhou et al., 1998). The less variable percentage CSF CD4 T cells expressing CD26 in patients carrying CCR5 Δ32 does, however, suggest that CCR5 Δ32 could influence differential recruitment of T cell subsets. Enhanced CD26 expression on peripheral blood lymphocytes was previously reported in patients with chronic progressive MS (Hafler et al., 1985). In the present study we found an increased percentage of peripheral blood CD4 T cells expressing CD26 even in patients with relapsing-remitting MS, supporting a possible role of this T cell subset in the pathogenesis of MS. CD26 is crucially involved in the activation of myelin-reactive CD4 T cells and may serve as a surface marker for Th1 cells (Annunziato et al., 1998; Reinhold et al., 1998). Th1 cells also express CCR5 and T cells expressing CCR5 commonly co-express CD26 (Bleul et al., 1997; Wu et al., 1997; Bonecchi et al., 1998; Loetscher et al., 1998; Qin et al., 1998; Sallusto et al., 1998; Balashov et al., 1999). Evidence supporting a direct role of CCR5 in the recruitment or activation of Th1 cells is, however, lacking.

CCR5 was expressed on a high percentage of CD8 T cells in patients with MS, and patients with MS have a higher percentage of peripheral blood CD8 T cells that express CCR5 than do healthy control subjects (Sørensen et al., 1999). CCR5 was also expressed on up to 100% of the CSF monocytes, and abundant CCR5 expression has been observed on phagocytic macrophages and microglia in active MS lesions (Balashov et al., 1999; Sørensen et

al., 1999). Monocyte recruitment is a central step in the pathogenesis of EAE and clinical disease activity is inhibited in monocyte depleted animals (Brosnan et al., 1981; Huitinga et al., 1990, 1995). Activation of microglia may also be involved in the pathogenesis of MS (Gay et al., 1997; Sriram and Rodriguez, 1997). These findings suggest that CCR5 signalling in CD8 T cells, monocytes, or microglia could be altered in MS patients carrying CCR5 Δ32.

The concentration of MMP-9 (gelatinase-B) is increased in CSF from patients with MS and correlates with the CSF leukocyte count (Gijbels et al., 1992; Paemen et al., 1994; Leppert et al., 1998). The results of the present study show that MMP-9 activity in CSF is also a risk factor for recurrent disease activity in MS. Although patients with MMP-9 activity in CSF had higher CSF leukocyte counts, IgG synthesis levels, CSF neopterin concentrations, more commonly enhancing lesions on MRI, and lower age, these variables were not associated with the risk of recurrent disease activity in the multivariate Cox regression model. This does not prove a causal relationship of MMP-9 activity and disease activity. The clinical effect of interferon-β1a treatment in MS was, however, recently reported to correlate with a decrease in the CSF leukocyte count, and a higher attack risk was observed in patients with high CSF leukocyte counts (Rudick et al., 1999). As treatment with interferon-β inhibits MMP-9 activity the observed decrease in CSF leukocyte counts after interferon-β1a treatment could, indeed, result from an effect on MMP-9 activity (Leppert et al., 1996; Stüve et al., 1996).

The present study provides evidence to suggest that signalling though the CCR5 chemokine receptor and MMP-9 activity, most likely by independent pathways, are involved in the pathogenesis of MS. Patients carrying the CCR5 Δ32 allele encoding a defective receptor had a lower risk of recurrent disease activity, and patients with MMP-9 activity in CSF had a higher risk of recurrent disease activity. In spite of the disease-modifying effect of the CCR5 Δ32 allele the carrier frequency of this allele was comparable in patients with MS and control subjects. This is in accordance with recent findings in EAE and a rat model of arthritis where different chromosomal loci were associated with disease onset, severity, and chronicity (Vingsbo-Lundberg et al., 1998; Butterfield et al., 1999). If our results can be confirmed in independent studies treatment with CCR5 antagonists or MMP-9 inhibitors may attenuate disease activity in patients with MS.

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ORIGINAL PAPER

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CC-chemokine receptor 5 polymorphism and age of onset in familial multiple sclerosis

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Abstract Multiple sclerosis (MS) is a common disease of the central nervous system characterized by myelin loss and progressive neurological dysfunction. An underlying genetic susceptibility plays a clear role in the etiology of MS, likely acting in concert with an undefined environmental exposure. Full-genome screenings in multiplex MS families have identified several susceptibility regions, supporting a polygenic model for MS. Among these regions, evidence for weak linkage was observed at 3p/3cen suggesting the presence of an MS gene(s) of modest effect. Encoded here are two chemokine receptors, *CCR5* and *CCR2B*. We examined the chromosome 3p21–24 region in 125 MS families (322 total affecteds and 200 affected sib-pairs), and performed genetic analyses of *CCR5* and *CCR2B* loci and two nearby markers (*D3S1289* and *D3S1300*) using both linkage- and association-based tests. No evidence of linkage to MS was observed for any of the tested markers. Affected relative-pair (SimIBD) and sib-pair analyses (ASPEX), and association testing (sib-TDT) for each locus were also not significant. However, age of onset was approximately 3 years later in patients carrying the *CCR5Δ32* deletion ($P=0.018$ after controlling for gender effects). Thus, chemokine receptor expression may be associated with differential disease onset in a subset of patients, and may provide a therapeutic target to modulate inflammatory demyelination.

Key words Human · Autoimmunity · Multiple sclerosis · Chemokine · Major histocompatibility complex

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Introduction

The pathogenesis of multiple sclerosis (MS) is complex and multifactorial with an underlying genetic susceptibility likely acting in concert with undefined environmental exposures (Ebers and Sadovnick 1994; Oksenberg et al. 1996). Using multiplex MS families collected in the USA, UK, and Canada, 10- to 15-cM whole-genome screens were completed and reported in 1996 (Ebers et al. 1996; Multiple Sclerosis Genetics Group 1996; Sawcer et al. 1996). Analysis of the data with both parametric (model-based) and nonparametric (model-free) statistical approaches revealed multiple susceptibility regions, supporting a polygenic model for MS, and significantly increased the list of potential candidate disease susceptibility genes. Evidence for weak linkage in the chromosomal region 3p/3cen was identified in the British screen with a maximum lod score around 1.3 (Sawcer et al. 1996). In follow-up studies using additional markers, similar results were obtained (Chataway et al. 1998), suggesting that this region may hold an MS gene(s) of modest effect. Furthermore, a meta-analysis of the linkage results from all three ge-

nome scans highlighted a nonparametric linkage (NPL) score >2 at 3p21 (S. Sawcer, personal communication). Encoded within this chromosomal region are two chemokine receptor genes, *CCR5*, a CC-type receptor that binds RANTES, macrophage inflammatory protein (MIP)-1α and MIP-1β, and *CCR2B*, a receptor for the monocyte attractants MCP-1, -2, -3, and -4. Independent of their suggestive location, *CCR5* and *CCR2B* are interesting MS candidate genes for several reasons. Aberrant expression of chemokines and chemokine receptors has been detected in both human and experimental central nervous system (CNS) demyelinating lesions (Hvas et al. 1997; Jiang et al. 1998; Sorensen et al. 1999), suggesting the involvement of chemokine-chemokine receptor interactions in disease pathogenesis. In addition, epidemiologic, migration, and cluster studies favor some role for an infectious agent in MS etiology (Johnson 1994). Chemokine receptors have been shown to mediate the entry of microorganisms into target cells (Alkhatib et al. 1996; Choe et al. 1996; Deng et al. 1996; Dragic et al. 1996), and to participate in the viral-mediated induction of type 1 cytokines (Ankel et al. 1998), potential mediators of the encephalitogenic response.

To clarify the genetic role of chemokine receptors in MS, we analyzed in detail the Chromosome (Chr) 3p21–p24 segment in a well-characterized dataset of multiple affected member families with the relapsing form of MS. Genetic analyses of common variants within coding regions of both *CCR5* and *CCR2B* loci, and of two nearby microsatellite markers, were performed using linkage- and association-based methodologies. Analysis was extended to a polymorphism within the 3′-untranslated region of the chemokine stromal-derived factor 1 (*SDF-1*) gene. *SDF-1*, genomically mapped to 10q11.1, is the principal ligand for CXCR4, the major coreceptor of T-tropic human immunodeficiency virus (HIV) strains (Bleul et al. 1996; Oberlin et al. 1996). Evidence for linkage to MS was not observed with any of the 3p21-associated markers in the MS families. Of interest, however, the mutant *CCR5* allele which carries a 32-bp deletion (termed *Δ32*), appears to confer a moderate, yet significant, delay in age of disease onset.

Materials and methods

Families

Rigorous clinical criteria were employed to identify and collect 125 Caucasian families with multiple cases of MS. In keeping with the variety of family structures seen in the general population, ascertainment was not restricted to a single family type. All affected members were interviewed and parental and ancestral information was recorded by countries of origin. All known ancestors were Caucasian and European in origin. To limit possible confounding effects of disease heterogeneity and misdiagnosis, families in which a primary progressive course was present in two or more members were excluded. An extensive system of error checking was employed to exclude all potential DNA sample and paternity problems in the dataset. Diagnostic criteria, ascertainment protocols, and clinical and demographic characteristics of the population are summarized elsewhere (Goodkin et al. 1991; Multiple Sclerosis Genetics Group 1998b). A previously reported *HLA* analysis in 98 of these families demonstrated linkage to the major histocompatibility complex (MHC) region (maximum lod score of 4.6), attributable to an *HLA-DR2* allelic association (Multiple Sclerosis Genetics Group 1998a). A second dataset of 299 sporadic Caucasian MS patients, with no record of affected relatives, was collected using the same clinical criteria. A sample of 147 healthy unrelated Caucasian individuals (primarily patient spouses) served as controls for association-based comparisons with MS patient groups.

Genotyping

White blood cells are routinely transformed to establish lymphoblastoid cell lines for all family members. High molecular-weight DNA was isolated using a standard desalting procedure. DNA samples were organized into genotyping keys, and 10 ng aliquoted into 96-well plates for 25–30 cycles of "hot start" PCR amplification using fluorescently-labeled oligonucleotide primers. PCR products (*D3S1289*, *D3S1300*, *CCR5*) were resolved by electrophoresis in 6% acrylamide denaturing gels on the ABI PRISM 373 Automated DNA Sequencer loaded with the ABI PRISM 672 GENESCAN 2.1 software for fluorescent scanning. Genotyper 2.0 was then used for peak calling and allele binning. The *CCR2B-64I* polymorphism was detected by PCR amplification, *Bsa*I digestion, and 4% Nusiev 3:1 electrophoresis (Kostrikis et al. 1998). The *SDF1–3′A* polymorphism was detected by PCR amplification, *Msp*I digestion, and 2% Nusiev 3:1 electrophoresis (Winkler et al. 1998). Generation of genotypes was performed blind to pedigree structure and to the clinical status of the family members. Data was formatted on MS-Excel and transferred to pedigree files drawn with Cyrillic 2.1 software, where Mendelian inheritance was automatically checked and confirmed. Allele information was then exported back into Excel and saved in ASCII files for direct downloading into the LAPIS data management system and storage in the PEDIGENE database (Haynes et al. 1995).

Statistical analysis

Linkage analysis included model-dependent and model-independent methods. Since the mode of MS inheritance is not known with certainty, both an autosomal dominant model assuming an MS disease allele frequency of 0.05, and an autosomal recessive model with an MS disease allele frequency of 0.02 were used for lod score analysis. Both of these models used phenotypic information on affected-only individuals, thus eliminating the information on all individuals not clinically definite for MS. Calculation utilized FASTLINK (Schaffer et al. 1994).

Because the dataset contained many affected sib-pairs and other affected relative pairs, enough information was available to perform genetic model-free analysis. SimIBD (Davis et al. 1996) allowed the inclusion of all affected relatives while not necessitating the assumption of genetic model. Sib-pair analysis (assuming a locus specific $\lambda_s = 3$) was completed using the sib-phase option of the ASPEX computer package (Hinds 1998). Family-based association studies were done using sib-TDT (Spielman and Ewens 1998). Marker allele frequencies were estimated from genotypic information derived from all unrelated married-in individuals in the dataset. These allele frequencies were compared to available data from a Caucasian control dataset and published frequencies. No significant differences were observed.

The effects of *CCR5*, *CCR2*, and *SDF1* phenotypes on age of disease onset in MS, adjusting for potential confounders such as sex and *HLA-DR2* status were analyzed in the multiplex families using linear mixed models (models with both fixed and random effects) as implemented in PROC MIXED in SAS version 6.12,

(SAS Institute, Cary, N.C.; Jennrich and Schlucter 1986; Laird and Ware 1982). These models are fit by restricted maximum likelihood and use random effects to take into account any correlation in age of onset between members of the same family, which would violate the independence assumption of ordinary linear regression models. This method also provides a measure of the statistical significance of the within-family correlation. If ages of onset show significantly more variation between than within families, a correlation of onset ages between related individuals within a family, in our case these are primarily siblings within a pair, is implied. Familial MS index cases and sporadic MS patient data analyses were performed using the linear regression module in SPSS version 9.0 (SPSS, Chicago, Ill.). In this study, age of onset was defined as the first episode of neurological dysfunction suggestive of demyelinating disease (Doolittle et al. 1990). In determining the age of onset, the patient was asked to recall his or her initial neurological symptom, including visual blurring due to optic neuritis, vertigo, Bell's palsy, tic douloureux, diplopia, Lhermitte's symptom, focal weakness, sensory symptoms, or paroxymal symptoms. For each case, medical records were reviewed to corroborate the reported age and minimize recall inaccuracy for early symptoms. Differences in phenotype frequencies between patient groups and between patient and control groups were compared using a chi-square test for heterogeneity.

Results

The familial dataset utilized in this study consists of a total of 891 genotyped individuals, including 322 affecteds and 200 affected sib-pairs. To take full advantage of the power of this dataset, a multi-analytical strategy was applied, including parametric lod score, SimIBD, sib-pair, and sib-TDT analyses as described in Materials and methods. To further test for heterogeneity and potential interactions between the candidate loci and the MHC at Chr 6p21.3, the MS family sample was divided into three groups based on *HLA-DR2* status. The first group consisted of families in which every affected individual carried at least one *DR2* allele (*DR2*+, $n=67$). The second group included families where some but not all affected individuals carried at least one *DR2* allele (*DR2*+/–, $n=28$). The third group was restricted to families where no affected individuals carried a *DR2* allele (*DR2*-, $n=30$). The implementation of this novel stratification scheme results in a loss of statistical power due to the reduced family sample size in each category but, on the other hand, may detect either interaction or independence between the loci under study and the MHC.

Two-point linkage results are shown in Table 1. All markers covering the candidate region provided negative lod scores with the highest total lod score of 0.88 ($\theta=0.10$) and 0.82 ($\theta=0.10$) for *D3S1300* in the *DR2*-subset, using the affected dominant or affected recessive models, respectively. The results of the SimIBD and sib-pair analyses are summarized in Table 2. Using both methods, no excess sharing of alleles among affected relatives reached significance, even after stratification by *DR2* status (data not shown). ASPEX-maximum lod scores were also not significant. Similarly, sib-TDT analysis failed to identify significant distortion in allele transmission, as did case control testing for all five loci using MS familial index cases and a sample of unrelated healthy Caucasian individuals for comparison (data not shown).

To determine whether any of the chemokine receptor genes or related loci might influence age of disease onset, all MS patients from the multiplex families were stratified into two categories for each locus according to whether they carried at least one copy of the mutant allele. Phenotypes for analysis therefore included: (1) *CCR5Δ32*+ and *CCR5Δ32*- (presence or absence of *CCR5Δ32* allele), (2) *CCR2-64I*+ and *CCR2-64I*- (presence or absence of *CCR2-64I* allele), and (3) *SDF-1A*+ and *SDF-1A*- (presence or absence of *SDF-1A* allele). No significant evidence for intraclass correlation for age of onset within this group of MS families was observed ($r=0.12$, $P=0.11$). Significant effects on age of onset in patients were present for both gender and *CCR5* phenotype variables. The estimated age of disease onset was 3.2 years later in male than in female

Table 2 Model-free linkage and association test results for all families

Marker	ASPEX Maximum lod score	simIBD *P*-value	sib-TDT χ^2	*P*-value
CCR2-64I	0.00	0.98	1.20	0.27
CCR5Δ32	0.02	0.63	0.29	0.59
D3S1289	0.00	0.59	8.97	0.79
D3S1300	0.18	0.63	11.11	0.52
SDF-1	0.00	0.89	2.18	0.14

Table 1 Two-point model-dependent linkage results. The map position for 3p21 markers is tel–*CCR2*-1 cM–*CCR5*-2 cM–*D3S1289*-9 cM–*D3S1300*-cen.*SDF-1* is located at 10q11.2 (*AD* affected dominant, *AR* affected recessive)

Marker	Max lod score							
	All families		All affecteds are *DR2*		Some affecteds are *DR2*		No affecteds are *DR2*	
	AD	AR	AD	AR	AD	AR	AD	AR
CCR2-64I	0.01	−0.01	−0.08	−0.02	0.15	−0.04	0.56	0.83
CCR5Δ32	0.16	0.13	0.08	0.07	0.21	0.25	−0.01	−0.01
D3S1289	0.02	−0.09	−0.08	−0.15	−0.01	−0.04	0.22	0.51
D3S1300	0.17	0.25	0.16	0.11	−0.10	−0.05	0.88	0.82
SDF-1	−0.30	−0.13	−0.11	−0.06	−0.13	−0.03	−0.04	−0.02

Table 3 The effects of gender and *CCRΔ32* phenotype on age of onset in familial and sporadic multiple sclerosis (MS)

Effect	*n*	Estimate (years)	95% confidence interval	*P*-value
Familial MS				
Gender (male): all affected[a]	302	3.2	1.0–5.3	0.004
CCR5Δ32: all affected (controlling for gender)	302	2.9	0.5–5.2	0.018
Gender (male): index cases only[a,b]	120	4.8	1.3–8.2	0.007
CCR5Δ32: index cases only[b] (controlling for gender)	120	5.1	1.7–8.4	0.004
Sporadic MS				
Gender (male)[a]	299	3.2	0.9–5.5	0.006
CCR5Δ32 (controlling for gender)	299	0.6	−2.0–3.3	0.644

[a] The estimated gender effect on age of onset observed in males is relative to the age of onset observed in female MS patients
[b] Only one individual from each family was used for analysis

MS patients ($P=0.004$; Table 3), and 2.9 years later in individuals carrying at least one copy of the *CCR5Δ32* allele ($P=0.018$) after adjustment for effects due to gender. This effect was independent of *DR2* status. Similar results were also observed for *CCR5* phenotypes when only MS index cases (one member from each family) were considered; however the effect was stronger (Table 3). In this group, the estimated age of disease onset was 4.8 years later in male than in female MS patients ($P=0.007$), and 5.1 years later in individuals carrying at least one copy of the *CCR5Δ32* allele ($P=0.004$). No significant effects on age of onset due to *CCR2B*, *SDF1*, or *HLA-DR2* were present.

The effect of *CCR5* phenotype on age of onset was also examined in an independent sample of 299 sporadic MS patients. As observed in the familial MS patient sample, the estimated age of onset was significantly later in sporadic MS males (3.2 years, $P=0.006$) compared to females (Table 3); however, age of onset effects due to *CCR5Δ32* were not present in this patient group. Hence, the observed effect of *CCR5* phenotype on age of MS onset was significantly different between sporadic and familial MS datasets, despite their similarities with respect to gender distribution and mean age of onset, both overall and in the male and female patients group considered separately (Fig. 1A,B). *CCR5* phenotype distributions were also similar in patient and control groups (Fig. 1C). However, fewer *CCR5Δ32* individuals were present in the sporadic MS samples compared to the index case group (19% vs 27%, respectively; $P=0.07$).

Due to the relatively low frequency of *CCR5Δ32* compared to wild-type alleles, a total of only six individuals within the sporadic and familial MS datasets (three in each group) were homozygous for the *CCR5Δ32* allele. Of these six individuals, two (one sporadic MS male and one familial MS female) had a later age of disease onset when compared to gender-specific means: 43 and 34 years of age, respectively. A test for interaction between gender and *CCR5* phenotype was also performed to determine whether gender-specific differences were present for *CCR5Δ32*. No significant results were observed in either the MS family data ($P=0.29$) or in sporadic MS datasets ($P=0.38$).

Discussion

This report indicates a possible genetic role for CCR5 in the pathogenesis of MS. A comprehensive investigation of the candidate Chr 3p21 region was performed using a large, well-characterized, and rigorously ascertained US-Caucasian familial MS dataset. While earlier genomic studies of MS with the UK dataset yielded suggestive results for markers located in this genomic region (Sawcer et al. 1996), we found no evidence of significant linkage or association with any of the 3p21 markers used in this study. However, assessment of the effect of *CCR5*, *CCR2B*, and *SDF-1* variants on age of disease onset revealed that familial patients carrying at least one copy of the *CCR5Δ32* mutated allele have a significant delay when compared to individuals with the wild-type phenotype. This observation provides support for the pathogenic significance of CCR5 and CC-type chemokines in autoimmune demyelination.

Genes encoding chemokines and chemokine receptors are attractive candidates to study in MS due to their key role in the physiology of inflammation, regulation of CNS trafficking across the blood-brain barrier, and their exploitation by certain microorganisms to initiate infection. Expression of CCR5 as well as its ligands, RANTES, MIP-1α, and MIP-1β in MS brain and cerebral spinal fluid has been consistently observed (Hvas et al. 1997; Sorensen et al. 1999). Chemokines and chemokine receptors have been implicated in the mechanisms underlying HIV pathogenesis. CCR5, for example, serves as coreceptor for the macrophage-tropic HIV-1 virus (Alkhatib et al. 1996; Deng et al. 1996; Dragic et al. 1996). The *Δ32* deletion causes a frameshift at amino acid 185, which results in a stop codon and premature truncation within the third extracellular domain, preventing expression of the receptor. Individuals homozygous for the *CCR5Δ32* mutation are highly resistant to HIV infection (Dean et al. 1996; Liu et al.

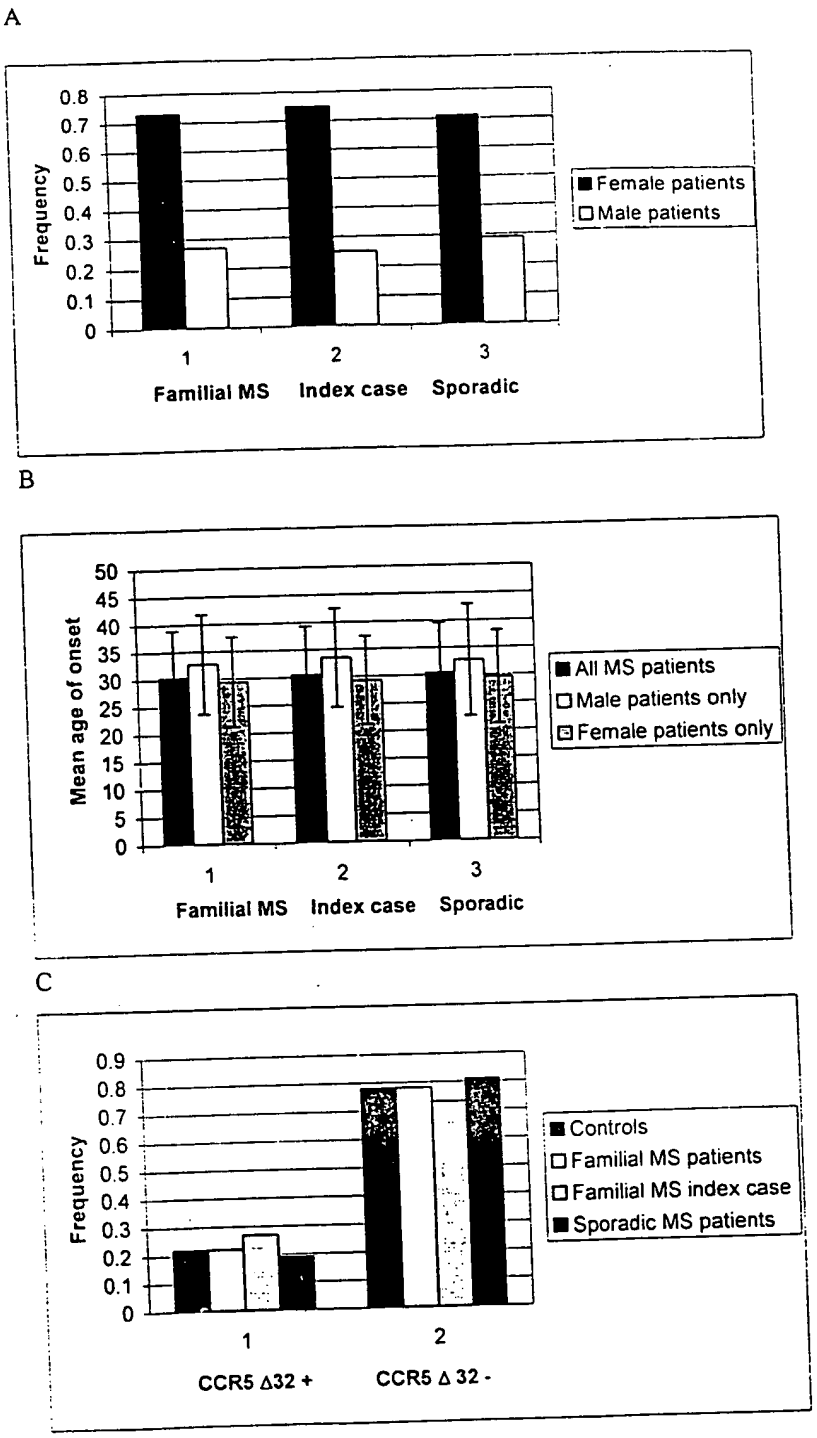

Fig. 1 **A** Gender distribution in familial ($n=302$), index cases ($n=120$). and sporadic ($n=299$) multiple sclerosis (MS) patient groups. The number of females and males in each group was very similar ($P>0.40$) for all comparisons. **B** Mean age of onset ($\pm$SD) in MS patient groups. The mean age of onset in familial (30.3 ± 8.6), index cases (30.5 ± 8.8), and sporadic (30.5 ± 9.1) patient groups was nearly identical. Male patients had a significantly later age of onset when compared to females in all three groups: familial (32.7 ± 9.1 vs 29.4 ± 8.2), index cases (33.5 ± 9.0 vs 29.3 ± 8.1), and sporadic (32.7 ± 10.2 vs 29.6 ± 8.5) patients. See Table 3 for corresponding *P*-values. **C** *CCR5* phenotype distributions in MS patient and control groups. Familial MS ($n=302$), index cases ($n=120$), sporadic MS ($n=299$), and control ($n=147$) groups were stratified into two categories for analysis: *CCR5Δ32+* and *CCR5Δ32-* (presence or absence of *CCR5Δ32* allele). Phenotype distributions in all MS patient groups were very similar ($P>0.40$) when compared to controls; however, fewer *CCR5Δ32+* individuals were present in the sporadic patient group

1996). In heterozygous individuals, infection can occur, but the rate of disease progression is lowered, with a typical delay of 2–4 years (Dean et al. 1996). Two additional chemokine polymorphisms recently observed to play a role in HIV pathogenesis – the *CCR2B* gene, which also maps to the 3p21.3 region, and the *SDF-1* gene, located in 10q11.2 – were also investigated in this study. No evidence for their involvement in MS was present in our datasets.

Mimicking MS demographics, a striking north to south gradient in the *Δ32* allele frequency has been reported, with the highest allele frequencies in Finnish and Mordvinian populations (16%), and the lowest in Sardinia (Martinson et al. 1997). Outside Europe, *Δ32* is seen at very low frequencies in populations from Asia, Saudi Arabia, India, and Pakistan, and it is virtually absent in native populations from sub-Saharan Africa, Oceania, and the Americas. This suggests that

the mutation has a recent and single origin in northeastern Europe (Stephens et al. 1998). In a recent study of 163 rheumatoid arthritis patients, disease activity, as measured by joint swelling and tenderness, morning stiffness, and IgM rheumatoid factor status (but not age of onset), was decreased in patients carrying the *CCR5Δ32* allele (Garred et al. 1998). Assuming an immunomodulatory role for the *CCR5Δ32* mutation, the provocative epidemiologic overlap between genetic resistance to AIDS and MS incidence may suggest that protection against initiation and progression of the autoimmune response provides a selective advantage to maintain the mutation in Caucasian groups (Stephens et al. 1998). An alternative interpretation of the demographic data could argue that *CCR5Δ32* might predispose to MS. However, this is in contrast to the results obtained in other studies. Bennetts and co-workers (1997) recently compared the frequency of *CCR5Δ32* in 120 Australian unrelated relapsing-remitting MS patients with a sample of 168 control individuals and found no evidence for either a protective or predisposing effect. Clinical variables such as age of onset were not examined. While neither family- nor case control-based approaches presented here demonstrated a significant MS association with either *CCR5* allele, this may be due in part to limitations imposed by sample size in each study, and also the reduced informativeness of the *CCR5* locus, and single nucleotide polymorphisms in general, to detect a modest association (Terwilliger and Weiss 1998; Xiong and Jin 1999).

The estimated age of onset in our familial dataset was 3.2 years later in male compared to female MS patients. This is expected, and has been previously observed in other studies including our own (Multiple Sclerosis Genetics Group 1998b). Surprisingly, the estimated age of onset was also significantly later in individuals carrying the *Δ32* allele, beyond the age effect attributed to gender alone. This was true both for all affected individuals and in a subset analysis that included only the MS index cases. No age-of-onset effects due to *CCR2B*, *SDF-1*, or *HLA-DR2* were present. In sporadic MS patients, a later age of onset in males was observed, but no effect due to *CCR5Δ32*. Perhaps there is an underlying genetic heterogeneity within the sporadic dataset, similar to that previously observed in familial MS in the *HLA* locus (Multiple Sclerosis Genetics Group 1998a). Such heterogeneity may dilute the statistical power to detect genetic effects of small magnitude. Alternatively, our data may reflect underlying genetic differences between the sporadic and familial datasets. While it is clear that both patient groups share a common *HLA-DR2* predisposition, additional MS susceptibility or disease-modifying loci within each group may be different. Our results emphasize the importance of considering clinical information in efforts to identify MS genes. Patient and control *CCR5* phenotype distributions were statistically indistinguishable, yet a significant effect on age of onset was observed for this locus in familial MS.

The development of inflammatory CNS lesions and detectable neurological deficits is likely the result of a multistep process that requires consecutive waves of activated lymphocytes crossing the blood-brain barrier. Reduced *CCR5* expression in heterozygous individuals, and its absence in homozygotes, could impair the efficiency of the homing process and the strength of the inflammatory response, delaying the expression of clinical signs. This hypothesis is in agreement with the observed increased expression of RANTES and MIP-1α in experimental allergic encephalomyelitis prior to and during the onset of clinical signs (Godiska et al. 1995; Karpus et al. 1995; Miyagishi et al. 1996), and during MS acute attacks (Sorensen et al. 1999). Because of the redundancy and overlapping molecules in the chemokine cascade, alternative pathways will eventually provide the necessary signaling and lymphocytic chemotaxis to initiate and perpetuate CNS inflammation. It is not surprising then that homozygosity for *Δ32* fails to protect against MS. The association between *CCR5Δ32* and delayed age of onset in MS may also result from linkage disequilibrium between the coding alleles and recently described polymorphisms within the *CCR5* promoter region which appear to influence gene expression and impact AIDS progression (Carrington et al. 1999; Martin et al. 1998; McDermott et al. 1998). Analysis of these and other polymorphisms in the receptor regulatory regions and ligands (Liu et al. 1999) in MS pathogenesis is warranted. Alternatively, although less likely, *CCR5* may be involved in the recognition of an undefined precipitating environmental factor, and as with the previous model, lower expression may affect the efficiency of the pathogenic process.

In conclusion, the age of onset in familial MS was found to be influenced by the *CCR5* genotype. This observation awaits confirmation in an independent dataset. It will be of interest to study prospectively the role of *CCR5*-associated polymorphisms in disease progression, CNS activity, and severity. In addition to age of onset, additional clinical phenotype-genotype analyses should be performed to determine whether the mutation also affects development of clinical signs of MS analogous to what is observed with rheumatoid arthritis (Garred et al. 1998). A major impact of the discovery of the HIV coreceptors is the potential availability of new therapeutic and preventive opportunities. Our data suggest that *CCR5* might prove to be an important target to modulate MS.

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Aberrant T cell migration toward RANTES and MIP-1α in patients with multiple sclerosis

Overexpression of chemokine receptor CCR5

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Summary

Trafficking of inflammatory T cells into the central nervous system (CNS) plays an important role in the pathogenesis of multiple sclerosis. The directional migratory ability of peripheral T cells is associated with interactions of chemokines with their receptors expressed on T cells. In this study, transmigration of peripheral T cells toward a panel of chemokines was examined in patients with multiple sclerosis and healthy individuals using Boyden chemotactic transwells. A significantly increased migratory rate preferentially toward RANTES and MIP-1α, but not other chemokines, was found in T cells obtained from multiple sclerosis patients as opposed to healthy individuals ($P < 0.001$). The migratory T-cell populations represented predominantly Th1/Th0 cells while non-migratory T cells were enriched for Th2-like cells. The study demonstrated further that aberrant migration of multiple sclerosis-derived T cells toward RANTES and MIP-1α resulted from over-expression of their receptors (CCR5) and could be blocked by anti-CCR5 antibodies. These findings have important implications for our understanding of the mechanism underlying aberrant T cell trafficking in multiple sclerosis.

Keywords: chemokines; multiple sclerosis; T cell migration

Abbreviations: CCR5 = Type 5 CC chemokine receptor; MIP-1α = macrophage-inflammatory protein-1α; MMP = matrix metalloproteinase; PBMC = peripheral blood mononuclear cells; PCR = polymerase chain reaction; PBS = phosphate-buffered saline; RANTES = normal T-cell expressed and secreted

Introduction

Multiple sclerosis is a chronic inflammatory and demyelinating disease of the CNS. One of the signature histopathological features of multiple sclerosis is the focal infiltration of inflammatory cells, predominantly $CD4^+$ Th1 cells, to the lesions (Hartung, 1993). There is increasing evidence to suggest the CNS inflammation is associated with aberrant trafficking of pro-inflammatory T cells across the blood–brain barrier, which is composed of tight tissue junctions to prevent peripheral immune cells and large molecules from entering the CNS (Hafler *et al.*, 1987; Chou *et al.*, 1992; Zhang *et al.*, 1994). Dubois and colleagues reported recently that immunization with myelin antigens failed to induce EAE (experimental autoimmune encephalomyelitis) in young knockout mice genetically deficient in matrix metalloproteinase (MMP)-9 (Dubois *et al.*, 1999), suggesting that access/trafficking of autoreactive T cells into the CNS is a crucial requirement for the development of EAE, an animal model for multiple sclerosis.

The ability of peripheral T cells to migrate is related to the expression of a number of T-cell surface molecules, including chemokine receptors and adhesion molecules. Many secretary molecules produced by T cells, including cytokines (e.g. γ-interferon) and MMP, also contribute to the migratory ability of T cells (Gijbels *et al.*, 1993; Hartung *et al.*, 1995; Hintzen *et al.*, 1995; Hvas *et al.*, 1997; Simpson *et al.*, 1998). In particular, new studies have begun to unfold evidence that chemottractants (chemokines) play a critical role in aberrant T cell trafficking in patients with multiple sclerosis (Balashov *et al.*, 1999; Sorensen *et al.*, 1999; Strunk *et al.*, 2000). To date, there are >40 chemokines and 10 chemokine receptors

that have been identified. Expression of some chemokine receptors, such as CCR5 (receptors for RANTES, MIP-1α and MIP-1β) and CXCR3 (receptors for IP-10 and MIG), is associated with Th1 pro-inflammatory cells, whereas Th2 anti-inflammatory cells preferentially express CCR3 (receptors for MCP-3, MCP-4 and RANTES), CCR4 (receptors for TARC and MDC) and CCR8 (Bonecchi *et al.*, 1998; Sallusto *et al.*, 1998; Zingoni *et al.*, 1998). CCR5 also serves as co-receptor for HIV-1 (Deng *et al.*, 1996; Dragic *et al.*, 1996). It was recently reported that some chemokine receptors, including CCR5 and CXCR3, were overexpressed among lesion-derived T cells and peripheral T cells in patients with multiple sclerosis (Balashov *et al.*, 1999; Sorensen *et al.*, 1999; Strunk *et al.*, 2000; reviewed in Zhang *et al.*, 2000). Elevated levels of chemokines, IP-10, RANTES and MIG were found in CSF obtained from patients with multiple sclerosis (Sorensen *et al.*, 1999). The studies were largely based on the binding assays in which antibodies were used to detect chemokines and chemokine receptors. These findings suggest that in multiple sclerosis, influx of pro-inflammatory T cells into the CNS may be associated with overexpression of chemokine receptors, which leads peripheral T cells to acquire aberrant trafficking properties toward certain chemokines produced at the site of pathology. However, it remains unclear as to which chemokine(s) or chemokine receptors are primarily responsible for aberrant trafficking of T cells in multiple sclerosis. This information is important for current research efforts to design specific antagonists for chemokine receptors, aiming to halt the influx of peripheral pro-inflammatory T cells into the CNS as a potential treatment for multiple sclerosis.

This study was undertaken to identify the chemokine(s) associated with aberrant T cell trafficking in multiple sclerosis using functional assays. We examined the migratory rate of peripheral T cells obtained from patients with multiple sclerosis and healthy individuals toward seven chemokines, including RANTES, MIP-1α, MIG, IP-10, IL-8, MCP-1 and MCP-3. The selection of these chemokines was based on potential association of some of them and their receptors with multiple sclerosis, as reported previously based on binding studies (Balashov *et al.*, 1999; Sorensen *et al.*, 1999). T cell migration study was performed using standard Boyden chemotactic chambers that allow directional migration of T cells toward chemokines of interest. We describe for the first time that peripheral T cells obtained from multiple sclerosis patients as opposed to healthy individuals exhibit a unique migratory pattern preferentially toward RANTES and MIP-1α but not towards other chemokines tested. The findings correlate with overexpression of CCR5, a chemokine receptor for both RANTES and MIP-1α, on peripheral T cells derived from multiple sclerosis patients compared with control T cells obtained from healthy individuals. The study has important implications for our understanding of the role of identified chemokines and chemokine receptors in aberrant trafficking of peripheral T cells in multiple sclerosis and the development of chemokine receptor antagonists as a potential treatment for multiple sclerosis.

Material and methods

Reagents

All chemokines and monoclonal antibodies to CCR1, CXCR3, CCR3 and CCR5 used in this study were purchased from PharMingen (San Diego, Calif., USA). Monoclonal antibodies to CD4 and CD8 were obtained from Becton Dickinson (San Jose, Calif., USA). Medium used for cell culture and migration experiments was RPMI-1640 supplemented with 10% heat-inactivated foetal calf serum (FCS) and L-glutamine, sodium pyruvate, non-essential amino-acids and 10 mM HEPES buffer (Hyclone, Logan, UT, USA).

Human subjects

Fifteen patients with multiple sclerosis (six males and nine females) were enrolled in this study. The diagnosis was made based on clinical manifestations and laboratory studies, and confirmed by MRI (Kurtzke, 1983; Poser *et al.*, 1983). All patients were characterized as having relapsing–remitting multiple sclerosis or chronic progressive multiple sclerosis for >2 years (average 6.2 years). The patients had not taken any immunosuppressive drugs for at least 3 months prior to the study. A group of 15 healthy volunteers (seven males and eight females of closely matched age) were included as control subjects. The protocol was approved by the Institutional Human Subjects Committee at Baylor College of Medicine. All subjects gave their informed consent to give blood samples for the study.

Enrichment of peripheral T cells

Peripheral blood mononuclear cells (PBMCs) were separated from freshly obtained blood specimens (within 2 h of blood draw) by a conventional Ficoll–Hypaque method (Zhang *et al.*, 1992). To enrich T cells, PBMC preparations were first incubated in Petri dishes at a concentration of 2×10^6 cells/ml for 2 h at 37°C to remove monocytes. Non-adhesive cells (enriched for T cells and some B cells) were subsequently passed through a nylon wool column to enrich the T cell population (loading volume of 2 ml, cell capacity 1.5×10^8) (Hathcock *et al.*, 1998). The nylon wool column was prepared by activating nylon wool fibres (Polysciences, Warrington, Penn., USA) with 1% HCl for 20 min and subsequently washing with water to obtain a neural pH. The column was equilibrated with RPMI-1640 and pre-warmed at 37°C for 30 min prior to use. T cells were then eluted

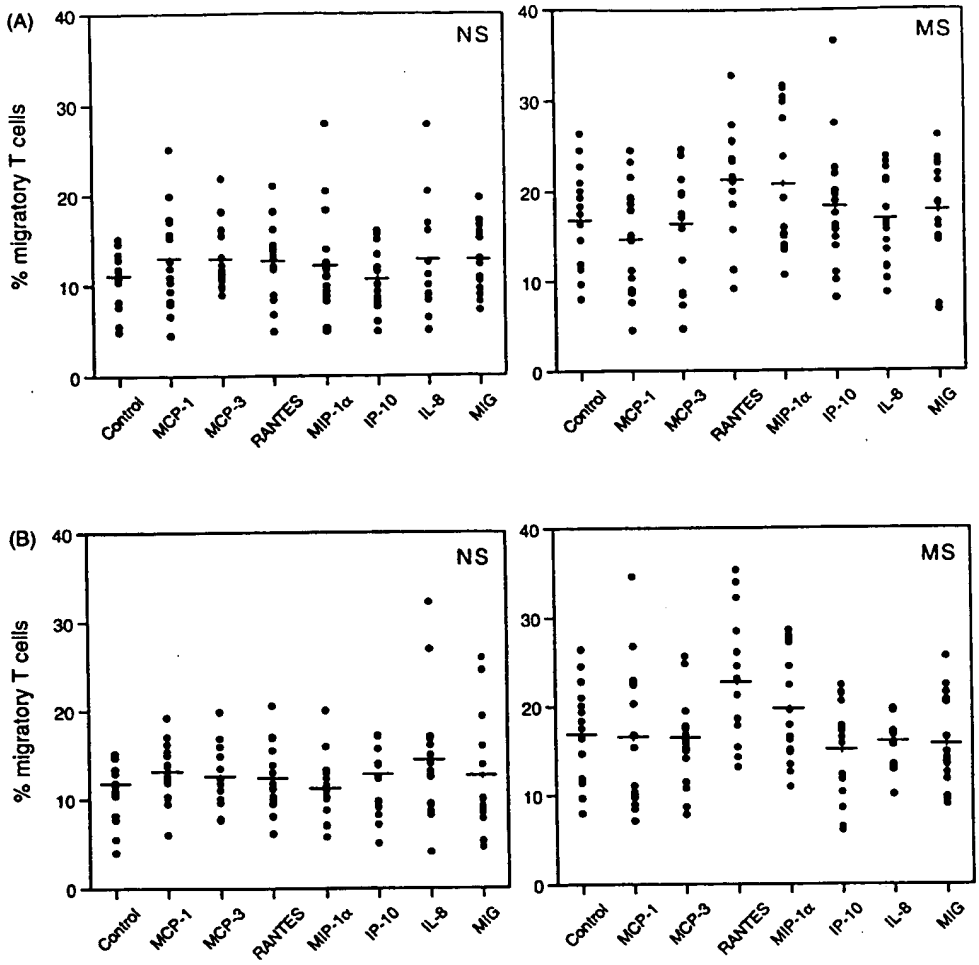

Fig. 1 Directional migration of peripheral T cells toward various chemokines. Peripheral T cells obtained from 15 multiple sclerosis patients and 15 healthy individuals were tested for migratory rate toward seven chemokines. T cell migration was performed in Boyden transwells in the presence and absence (control) of respective chemokines added in the lower chamber. Data are given as percentage T cells migrated after 3 h. **(A)** Rate of T cell migration toward respective chemokines at a concentration of 0.1 ng/ml. **(B)** Rate of T cell migration toward chemokines at a concentration of 1 ng/ml. Statistical significance was calculated by paired *t*-test. MS = multiple sclerosis patients; NS = normal subjects.

with 15–20 ml RPMI-1640 medium. The purity of T cells was >90% as determined by flow cytometry using an anti-CD3 monoclonal antibody (Becton Dickinson).

T cell migration study

T cell migration was performed in micro-transwells of a 5 μm pore size membrane (48-well Boyden chamber, Neuro Probe, Cabin John, Md., USA) (Pilaro *et al.*, 1990; Taub *et al.*, 1995). T cell preparations were added at 50 000 cells/well in the upper chambers and were incubated at 37°C for 3 h. The experimental conditions were pre-determined in a series of pilot experiments. The migratory T cells in the lower chambers were numerated microscopically in three different fields. The data are expressed as the mean number of migratory T cells as a percentage of the total number of cells taken ± standard deviation.

Separation of migratory and non-migratory T cells by Boyden transwells

The same experimental conditions as described above were used for T cell migration. Briefly, T cells derived from multiple sclerosis patients and control subjects were allowed to migrate in the presence of 1 ng/ml of RANTES using large transwells of a 5 μm pore size membrane (10-well Boyden chamber, Neuro Probe). These transwells provided a large surface area to accommodate more T cells for migration. All experiments were performed under sterile

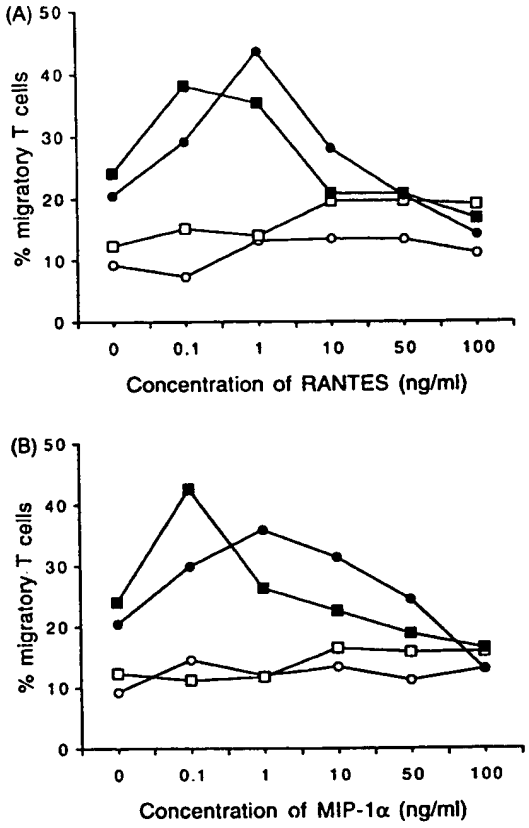

Fig. 2 Migratory rate of peripheral T cells in response to (A) RANTES and (B) MIP-1α. In a representative experiment, T cells derived from two multiple sclerosis patients (MS) and two normal subjects (NS) were tested for migratory rate in response to RANTES and MIP-1α at concentrations ranging from 0.1 to 50 ng/ml. Experiments were performed in Boyden transwells as described in the legend to Fig. 1. Data are given as the percentage of migratory T cells in relation to the total number of T cells. Open circles = NS1; open squares = NS2; filled circles = MS1; filled squares = MS2.

conditions as the resulting T cells would be recovered and cultured for cytokine analysis. After incubation for 3 h, migratory and non-migratory T cells were collected from the lower and upper chambers, respectively. The resulting T cells were washed and plated out at 20 000 cells/well in the presence of phytohaemagglutinin (PHA) (1 μg/ml) to induce cytokine production (Kozovska *et al.*, 1999). Culture supernatants were collected 48 h later and were subject to cytokine analysis by enzyme-linked immunosorbent assay (ELISA).

Semi-quantitative measurement of mRNA by polymerase chain reaction (PCR)

Total RNA was prepared from purified T cells using RNeasy kit (Qiagen, Valencia, Calif., USA), and cDNA was synthesized from 1 μg of isolated RNA using random hexamer and Superscript reverse transcriptase (Gibco, Grand Island, NY, USA), according to the manufacturer's instructions. PCR was performed using paired 5′ and 3′ primers specific for CCR5 or β-actin. The conditions used for PCR were as follows: denaturing at 94°C for 5 min; thermal cycling at 94°C for 30 s, 57°C for 30 s, 72°C for 45 s; and an additional extension at 72°C for 7 min after the last thermal cycle. β-actin was employed as an internal control for PCR amplification. The primer sequences are as follows: CCR5 sense primer 5′-TATTATACATCGGAGCCC, antisense primer 5′-GGTGTAATGAAGACCTTC; β-actin sense primer 5′-AAGTACTCCGTGTGGATCGG, antisense primer 5′-AAAGCCATGCC-AAACTCATC. Thirty PCR cycles were performed to ensure that PCR amplification did not reached the plateau. The amplified PCR products were separated on 1% agarose gel and transferred to a positively charged nylon membrane (Boehringer Mannheim, Indianapolis, Ind., USA) using vacuum blot (Bio-Rad, Hercules, Calif., USA) at 5 mmHg for 90 min. DNA was fixed onto the membrane by 3 min exposure to UV crosslinking and prehybridized at 68°C for at least 1 h. Poly(A) (0.1 mg/ml) was added to prehybridization solution [5× standard saline citrate (SSC), 1% blocking solution, 0.1% *N*-lauroylsarcosine, 0.02% sodium dodecyl sulphate (SDS)] to reduce non-specific binding of the probe to non-target DNA. Hybridization temperature and washing conditions were optimized according to the probes to ensure a stringent hybridization condition. Hybridization was carried out in a buffer containing 5× SSC, 1% blocking solution, 0.1% *N*-lauroylsarcosine, 0.02% SDS, 0.3 pmol/ml digoxigenin-labelled probe for 6 h. The detection of DNA hybrid products was performed using the Digoxigenin Luminescent Detection Kit according to the manufacturer's instructions (Boehringer Mannheim). The membrane was then exposed to X-ray film for 15–30 min at room temperature. The intensities of PCR products were quantified using a Gel Doc 1000 scanning densitometer (Bio-Rad). The mRNA expression of CCR5 was analysed relative to that of β-actin in each sample, and expressed as the ratio of CCR5 to β-actin in percentile (Hong *et al.*, 1999).

Flow cytometry

PBMCs were stained with the following monoclonal antibodies in pairs for direct dual staining in flow cytometric analysis: phycoerythrin (PE)-conjugated anti-CD4 and anti-CD8 antibodies/fluorescein isothiocyanate (FITC)-conjugated antibodies to CCR1, CXCR3, CCR3 and CCR5. FITC-anti-IgG1/PE-anti-IgG2a (simulset control) to detect background staining (Becton Dickinson). PBMCs were washed twice in Eppendorf tubes by the addition of 1 ml FACS buffer [phosphate-buffered saline (PBS) containing 5% foetal calf serum (FCS) and 0.01% sodium azide] and centrifugation at 2300 r.p.m. for 2 min at 4°C. The cells were then resuspended and stained with 10% of respective conjugated antibodies for 30 min on ice. After two washes the cells were resuspended in 300 μl FACS buffer and analysed by flow cytometry using a FACScan (Becton Dickinson) with gates set to read the total lymphocyte population.

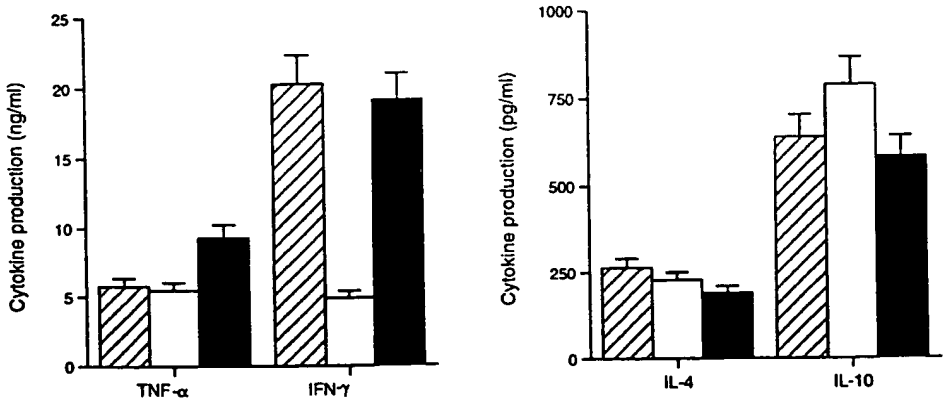

Fig. 3 Cytokine profile of migratory (black bars) and non-migratory (white bars) T cells (striped bars = T cells prior to migration). Peripheral T cells derived from three multiple sclerosis patients were allowed to migrate in large transwells and separated according to their migratory property toward RANTES. The resulting migratory and non-migratory T cells were recovered, washed and cultured at 20 000 cells/well in the presence of PHA (1 μg/ml). Supernatants were collected 48 h later and measured for concentrations of IL-4, IL-10, TNF-α and γ-IFN by ELISA. Data are presented as the mean cytokine concentrations of three specimens ± standard deviation. Standard deviation within each specimen analysed was <12%.

Statistical analysis

A Student's *t*-test was used to analyse the statistical significance of the differences in the migratory rate and the expression of CCRs between the study groups. A P value of <0.05 was considered significant.

Cytokine measurement

The cytokine production of the migratory and non-migratory T cells separated using Boyden transwells was examined in culture supernatants after they were challenged with phytohaemagglutinin-P (PHA-P) (Kozovska *et al.*, 1999). Supernatants were collected for the measurement of interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), interleukin-4 (IL-4) and IL-10, 48 h after stimulation. Culture supernatants were diluted 1 : 4 with PBS prior to assays. Cytokines were determined quantitatively using ELISA kits obtained from PharMingen. The kits were used according to the manufacturer's instructions. Briefly, 96-well microtitre plates (Nunc, Maxisorp) were coated overnight at 4°C with 2 μg/well of respective mouse capturing monoclonal antibodies in PBS. Wells were then blocked at 37°C for 2 h with 2% bovine serum albumin (BSA)–PBS and washed three times with cold washing solution, containing 0.02% Tween-20. Fifty microlitres of each sample and its control were added to the adjacent wells and incubated for 2 h at ambient temperature with 50 μl of a biotinylated detecting antibody (0.25 μg/ml of each monoclonal antibody) in 2% BSA–PBS–Tween-20. Plates were washed and incubated for 30 min with streptavidin-conjugated horseradish peroxidase. One hundred microlitres of 0.0125% TMB (tetramethylbenzidine) and 0.008% H_2O_2 in citrate buffer was used as substrate, and colour development was stopped using 100 μl of 1 N HCl. The concentration of each cytokine in a given sample was calculated using a double standard curve of corresponding recombinant cytokine (PharMingen) in each ELISA plate, which also served as a quality control. The detection limit for all cytokine measurements was <35 pg/ml in all assays.

Results

Directional migration of peripheral T cells obtained from multiple sclerosis patients and healthy individuals toward various chemokines

A group of 15 patients with either relapsing–remitting (n = 11) or chronic progressive multiple sclerosis (n = 4) was enrolled for this study. The mean expanded disability scale score (EDSS) of the patients was 3.5 with a mean disease duration of 6.2 years. Fifteen healthy volunteers were included as control subjects. Peripheral T cells purified from PBMCs were examined for random spontaneous migration (in the absence of chemokines) and directional migration toward selected chemokines using standard Boyden transwells. As shown in Fig. 1, T cells derived from multiple sclerosis patients exhibited a significantly increased rate of random migration (16.2%) in the absence of chemokines compared with that obtained from control subjects (11.2%). When tested with a panel of seven chemokines, including RANTES, MIP-1α, MIG, IP-10, IL-8, MCP-1 and MCP-3, multiple sclerosis-derived T cells displayed a characteristic migration pattern that differed from that of control T cells derived from healthy individuals (Fig. 1).

Multiple sclerosis-derived T cells exhibited a significantly increased migratory rate preferentially toward RANTES and MIP-1α ($P < 0.001$) but not other chemokines. The pattern of preferential T cell migration toward RANTES and MIP-1α remained largely unchanged when the chemokines were

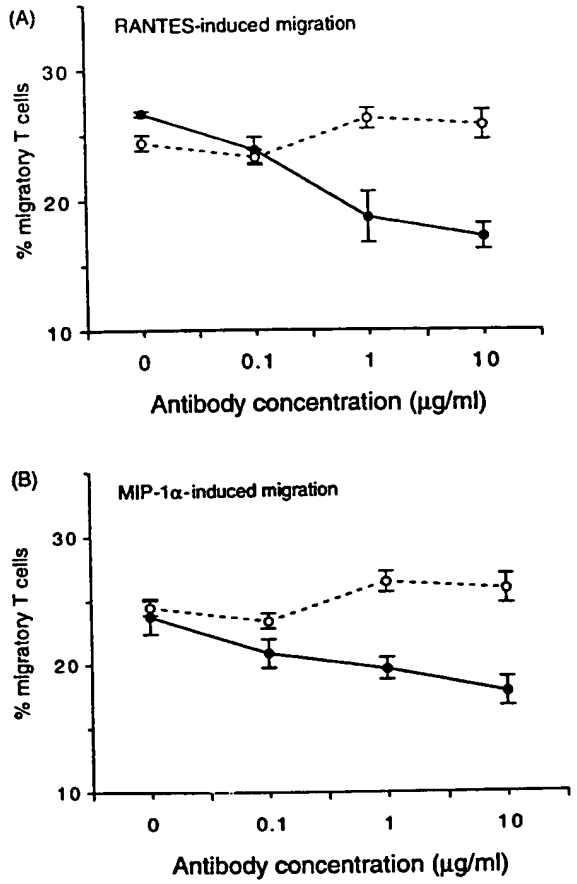

Fig. 4 Blocking of chemokine-induced migration of multiple sclerosis-derived T cells by an anti-CCR5 antibody. Multiple sclerosis-derived T cells were first incubated with an anti-CCR5 antibody (filled circles, solid lines) or an isotype-matched control antibody (specific for myelin basic protein) (open circles, dotted lines), at the indicated concentrations for 15 min. The migratory rate of the resulting T cells was measured in the presence of (A) RANTES (1 ng/ml) or (B) MIP-1α (0.1 ng/ml), respectively, in traswells under the same experimental conditions as described in the legend to Fig. 1. Data are expressed as the percentage of migratory T cells in relation to the total number of T cells loaded.

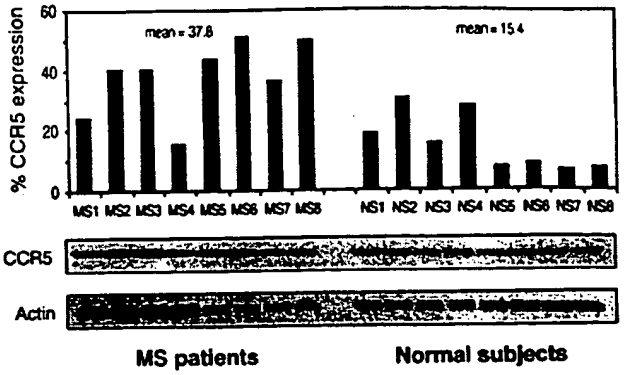

Fig. 5 Levels of mRNA expression for CCR5 in T cells derived from multiple sclerosis patients and normal subjects. T-cell preparations were derived from eight multiple sclerosis patients (MS) and eight normal subjects (NS) and subjected to analysis for the expression of mRNA for CCR5 by semi-quantitative PCR. Levels of mRNA expression for CCR5 were relative to that of β-actin as an internal control in the same T-cell preparations. The chemiluminescent intensities of hybridized products were quantified using a densitometer. The percentage of relative expression of mRNA for CCR5 is calculated as follows: (expression of mRNA for CCR5/expression of mRNA for β-actin)×100%.

used at 0.1 and 1 ng/ml (Fig. 1A and B), respectively. These concentrations were found to be optimal concentrations in pilot experiments and within the concentration range of the chemokines found previously in serum and cerebrospinal fluid specimens obtained from patients with multiple sclerosis (Balashov *et al.*, 1999; Sorensen *et al.*, 1999). In contrast, when used at similar concentrations, the same chemokines, including RANTES and MIP-1α, did not significantly alter the migratory rate of control T cells derived from healthy individuals (Fig. 1A and B). Peripheral T cells derived from selected multiple sclerosis patients and control subjects were further characterized for migration toward RANTES and MIP-1α in a dose-dependent manner. As shown in Fig. 2, the most effective concentrations of RANTES and MIP-1α were between 0.1 and 1 ng/ml for multiple sclerosis-derived T cells, while control T cells exhibited minimal migration toward the chemokines used at the same concentrations. Taken together, the findings indicate that RANTES and MIP-1α but not other chemokines, selectively alter the migratory property of multiple sclerosis-derived T cells.

Cytokine profile of migratory T cells induced by RANTES

It was of interest to delineate whether RANTES selectively facilitated migration of a subset(s) of multiple sclerosis-derived T cells and whether the migratory T cells exhibited a characteristic cytokine profile. To address these issues, peripheral T cells were separated into migratory and non-migratory T cell populations by transwell migration toward RANTES. As illustrated in Fig. 3, in the three multiple sclerosis-derived T cell specimens analysed, the migratory T cells derived from multiple sclerosis patients were predominantly Th1/Th0 cells while non-migratory T-cell populations were enriched for Th2-like cells. The non-migratory T cell populations produced predominantly IL-10 and substantially less γ-interferon compared with T cells prior to migration, suggesting that RANTES selectively facilitated transwell migration of T cells of predominantly Th1/Th0 phenotype.

Overexpression of CCR5 in peripheral T cells obtained from multiple sclerosis patients

The observation that RANTES and MIP-1α, the ligands for CCR5, selectively altered the migratory property of multiple sclerosis-derived peripheral T cells prompted us to investigate

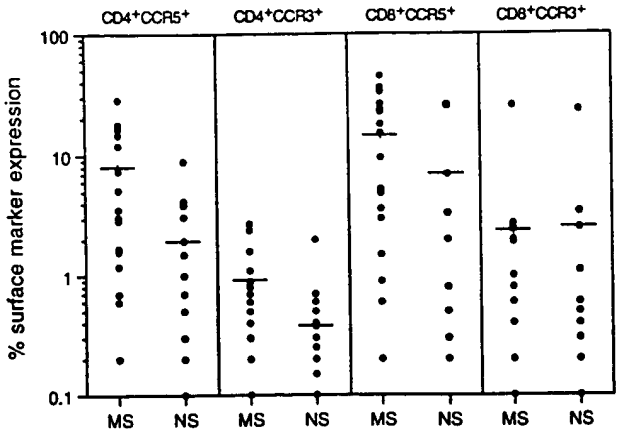

Fig. 6 Surface expression of CCR3 and CCR5 on PBMCs obtained from multiple sclerosis patients and healthy controls. PBMC specimens obtained from 15 multiple sclerosis patients and 15 normal subjects (NS) were examined for the expression of CCR5 and CCR3 among $CD4^+$ and $CD8^+$ T-cell populations. Cells were double stained with the conjugated antibody pairs as indicated. Percentage of positive T cells was determined by flow cytometry.

further whether the selective effect of the two chemokines on T cell migration was related to potential overexpression of their receptors (CCR5) on multiple sclerosis-derived T cells. To this end, we first examined whether transmigration of multiple sclerosis-derived T cells induced by RANTES and MIP-1α could be blocked by an anti-CCR5 antibody. As shown in Fig. 4, the addition of an anti-CCR5 antibody, but not an isotype-matched control antibody, substantially inhibited transwell migration of multiple sclerosis-derived T cells toward RANTES and MIP-1α in a dose-dependent manner. As both RANTES and MIP-1α are known to interact with additional chemokine receptors other than CCR5, e.g. CCR3 and CCR1, it is not surprising that the blocking of CCR5 resulted in partial but not complete inhibition of T cell migration.

Next, we examined mRNA expression of CCR5 in peripheral T cells obtained from multiple sclerosis patients and control subjects by semi-quantitative PCR. The results revealed that mRNA levels were significantly higher in multiple sclerosis-derived T cells (37.8%) that were not stimulated, compared with control T cells (15.4%, $P < 0.01$) (Fig. 5). Furthermore, as illustrated in Fig. 6, when analysed for the surface expression of CCR5, multiple sclerosis-derived T cells overall exhibited a significantly higher expression of CCR5 in both $CD4^+$ and $CD8^+$ T-cell populations than that found in control T cells obtained from healthy individuals ($P < 0.001$). $CD4^+$ T-cell preparations derived from multiple sclerosis patients were significantly enriched for $CCR3^+$ T cells compared with control T cells (Fig. 6). In a series of additional experiments, we examined the expression of CCR1 and CXCR3 in T cells derived from 10 multiple sclerosis patients and eight healthy individuals under the same experimental conditions. The expression level of CCR1 did not differ significantly between multiple sclerosis patients and control subjects (mean 2.6 versus 2.4%, $P > 0.1$) while CXCR3 expression was only slightly higher in multiple sclerosis-derived T cells (16.6%) compared with control T cells (14.8%, $P > 0.1$) (data not shown). Taken together, these findings suggest that aberrant migration of multiple sclerosis-derived T cells toward RANTES and MIP-1α is associated with significant overexpression of their receptors (CCR5).

Discussion

The recent discovery of chemokines and chemokine receptors has begun to unfold the molecular basis of how T cells are directed to the site of inflammation. The role of chemokines and chemokine receptors is particularly important in multiple sclerosis, in which myelin-destructive inflammation occurs behind the blood–brain barrier and is related to influx of peripheral pro-inflammatory T cells into the CNS. In this respect, the study described here provides direct evidence, based on functional assays, that two chemokines, namely RANTES and MIP-1α which share the same receptor (CCR5), are preferentially involved in directional migration of peripheral T cells in patients with multiple sclerosis. A concentration of 0.1 ng/ml of RANTES and MIP-1α is sufficient to induce T cell migration and mRNA synthesis of MMP-9. This concentration of RANTES and MIP-1α was found previously in CSF obtained from multiple sclerosis patients but not in control subjects (Balashov *et al.*, 1999; Sorensen *et al.*, 1999). Furthermore, the observation that peripheral T cells derived from multiple sclerosis patients exhibited a significantly higher random migratory rate than those from healthy individuals is consistent with the previous findings that multiple sclerosis peripheral T cells, including myelin-reactive T cells, undergo *in vivo* activation and have migratory advantages (Hafler *et al.*, 1987; Allegretta *et al.*, 1990; Chou *et al.*, 1992; Zhang *et al.*, 1994). The study described here provides experimental evidence that RANTES selectively facilitated migration of Th1/Th0-like cells. The finding is particularly relevant to the understanding of the mechanism underlying trafficking of pro-inflammatory T cells into the CNS and the site of pathology as the pro-inflammatory cytokines produced by Th1 cells, such as TNF-α and γ-interferon, are associated with clinical activity in multiple sclerosis (Panitch *et al.*, 1987; Sharief *et al.*, 1991).

It was demonstrated in this study that aberrant migration of multiple sclerosis-derived T cells preferentially toward RANTES and MIP-1α is attributable to the overexpression of CCR5. Overexpression of CCR5 in PBMCs obtained from patients with multiple sclerosis was also reported by other investigators based on binding assays (Balashov *et al.*, 1999; Sorensen *et al.*, 1999; Strunk *et al.*, 2000). It is important to note that although several other chemokines (e.g. IP-10) and chemokine receptor expression (e.g. CXCR3) are also elevated in the CSF, peripheral blood and even in post-mortem multiple sclerosis lesions as reported previously, they

do not directly alter the migratory property of multiple sclerosis-derived T cells in the functional studies described here. It suggests that local inflammatory processes may result in the production and accumulation of a variety of chemokines, but not all chemokines produced at the site of inflammation are necessarily associated with aberrant T cell trafficking in multiple sclerosis, strengthening the importance of the finding described here based on the functional assays. On the other hand, it is conceivable that CCR5 may not be the only chemokine receptor involved in the aberrant T cell migration in multiple sclerosis. This possibility is supported by a previous report that the lack of CCR5 expression fails to protect against multiple sclerosis (Bennetts *et al.*, 1997) and by the observation that blocking of CCR5 did not result in complete inhibition of T cell migration, as described here. As some of the chemokines, including RANTES and MIP-1α, share different receptors, blocking of a single chemokine receptor is not sufficient to abolish T cell migration.

The study suggests that overexpression of CCR5 is critical to aberrant migration of peripheral T cells toward the site of inflammation in multiple sclerosis. It is probable that the observed overexpression of CCR5 in multiple sclerosis patients may be triggered by certain events associated with multiple sclerosis. Among many possibilities, viral infection is of particular relevance. There is increasing evidence to suggest that human herpes virus (HHV)-6 may be associated with multiple sclerosis. Cell-free viral DNA and elevated antibody titers to HHV-6 were found in CSF and serum specimens of patients with multiple sclerosis but not in healthy individuals (Soldan *et al.*, 1997). It was reported recently that an open-reading frame within HHV-6, designated as U12 gene, encodes a functional receptor for RANTES (Isegawa *et al.*, 1998). Although it is unclear at this time whether re-activation of HHV-6 in multiple sclerosis patients is responsible for the overexpression of CCR5 per se or the induction of a viral chemokine receptor similar to CCR5, the finding opens new possibilities in the search for a link between viral infection and the autoimmune mechanism involved in multiple sclerosis.

Acknowledgement

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ELSEVIER

The CCR5 Deletion Mutation Fails to Protect Against Multiple Sclerosis

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ABSTRACT: Recent advances in the understanding and identification of chemokines and their receptors have provided evidence for their consideration as candidate loci with respect to genetic susceptibility/resistance to MS. Increased levels of the chemokine, macrophage inflammatory protein (MIP)-1α, have been demonstrated in the cerebrospinal fluid of both patients with MS and mice with EAE, and anti-MIP-1α antibodies have been shown to prevent EAE.

Recently, a common deletion mutation in the gene for the major receptor for MIP-1α, chemokine receptor 5 (CCR5) has been described. Homozygotes for the mutation fail to express this receptor. Moreover, homozygotes are highly protected against HIV infection; this has potential implications for the cell entry of infectious agents in other multifactorial diseases where a viral component may be involved. In view of these aspects, a group of 120 unrelated Australian relapsing/remitting MS and 168 unrelated control subjects were screened for the CCR5Δ32 mutation. There was no significant difference in the allele frequency of CCR5Δ32 gene between the MS patients (0.1125) and the control population (0.0921). The presence of two CCR5Δ32 homozygotes in the MS patients indicates that the absence of CCR5 is not protective against MS. These data suggest that CCR5 is not an essential component in MS expression, though this may be due to redundancy in the chemokine system where different chemokine receptors may substitute for CCR5 when it is absent. *Human Immunology 58, 52–59 (1997).* © American Society for Histocompatibility and Immunogenetics, 1997. Published by Elsevier Science Inc.

INTRODUCTION

The results of the recent complete genome screens in Multiple Sclerosis (MS) families [1–3] support the concept that MS is the result of the epistatic interaction of several genes [4, 5]. The failure of these screens to identify genetic regions of greater importance than the major histocompatibility complex (MHC) has made it clear that the path ahead is a difficult one. Whilst more detailed genome screening will continue, it is clear that the continued examination of candidate loci is important. Candidate loci are those which contain genes or gene complexes which control processes of proven or likely involvement in the pathogenesis of the disease. As the basic understanding of the autoimmune inflammatory response expands, polymorphic genes involved in this process become possible candidates for genetic susceptibility either acting alone or in combination with alleles of other important loci, such as the MHC and the T cell receptor (TCR) genes.

The migration of cells into sites of inflammation is stimulated by chemoattractant molecules, termed chemokines. Recent advances in the understanding and identification of chemokines and their receptors has provided evidence for their consideration as candidate loci in an autoimmune disease such as MS. The entry of T cells, across the blood-brain barrier, into the central nervous system (CNS) is one of the first steps in the animal model of MS, experimental autoimmune encephalomyelitis (EAE) and probably in MS itself. Increased levels of the chemokine, macrophage inflammatory protein (MIP)-1α, have been demonstrated in the cerebrospinal fluid (CSF) of patients with MS in relapse [6], and elevated MIP-1α in the CNS correlated with clinical disease in a relapsing/remitting model of EAE using SJL/J mice immunized with myelin proteolipid protein (PLP) [7]. Moreover, administration of anti-MIP-1α in these animals prevented the development of both acute and relapsing paralytic disease by preventing the infiltration of mononuclear cells into the CNS [7]. In another study of

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EAE, there was shown by mRNA analysis to be an induction in the spinal cord of a number of chemokines including: RANTES, MIP-1α, MIP-1β, and monocyte chemotactic protein (MCP), one to two days before clinical signs were apparent [8]. This suggests that the chemokine pathway is an important component in the aetiology of EAE and MS and blocking this pathway may prevent the expression of disease.

The major receptor for MIP-1α (and MIP-1β and RANTES) is the C-C chemokine receptor 5 (CCR5). The human CCR5 gene has been mapped to chromosome 3p21 [9] and is clustered with a number of other chemokine receptor genes. Recently, a 32 base pair deletion mutation in the CCR5 gene (*CCR5Δ32*) has been described which results in the failure to express the receptor on the surface of cells of *CCR5Δ32* homozygotes [10]. This mutation is surprisingly frequent in Caucasian populations of western European origin with heterozygote and homozygote frequencies of nearly 18% and 1%, respectively, in France and Belgium [11], the United States [9, 12] and Australia [13]. Considerable interest in this receptor and its mutation has resulted from the discovery that CCR5 acts as a co-receptor, with CD4, for the entry of the human immunodeficiency virus (HIV)-1 into human cells. Moreover, *CCR5Δ32* homozygotes are highly protected against HIV infection and heterozygotes may have slower disease progression [9] indicating potential for quite marked biological effects of this mutation.

An alternative hypothesis for the involvement of the chemokine system and a possible effect of the CCR5Δ32 mutation in MS, is the growing body of evidence which indicates that chemokine receptors have a broader role in the binding and entry of micro-organisms into host cells. This has been shown with both primate and non-primate lentiviruses [14] and with *plasmodium vivax* infection [15]. In addition, three herpes viruses, H. Saimiri, Human CMV [16] and Kaposi's sarcoma-associated herpes virus [17], have been found to have functional chemokine receptor genes. Given the known environmental component in MS [18], the absence of a chemokine receptor could be protective against infection with a putative MS associated virus.

In view of the likely involvement of the chemokine MIP-1α in T-cell mediated inflammation in the CNS, and that the deletion mutation in the gene for its receptor has demonstrated biological effects, we examined the CCR5 genotype in 120 unrelated Australian MS patients with relapsing/remitting MS and compared them with ethnically matched controls. This study also sought to investigate whether there was an interaction between the *CCR5Δ32* mutation, HLA phenotypes and T-cell receptor Vβ germline polymorphisms. While the chromosomal location of the CCR5 gene, 3p21 [9] was not in a region clearly identified by one of the three genome screens [1–3], the gene nevertheless has immunological importance. The homozygous form of the *CCR5Δ32* mutation may be protective against MS which, given the frequency of *CCR5Δ32* homozygotes, would not be easily detected in multiplex studies. If the homozygous form of the *CCR5Δ32* mutation is protective and CCR5 is shown to be essential for MS expression, this may open up a new range of therapeutic agents aimed at blocking the CCR5 receptor. This strategy is currently being developed for HIV therapy [19].

MATERIALS AND METHODS

MS Patients and Control Subjects

A group of 120 unrelated Australian MS patients with relapsing/remitting disease were selected by established criteria. Patients all had clinically definite or laboratory-supported definite MS according to the criteria of the Poser Committee [20], with a disease duration of between 2 and 15 years and disability on the Kurtzke expanded disability status scale (EDSS) from 2 to 5 [21]. A normal control panel of 168 unrelated subjects was gathered from hospital staff and unrelated partners of MS subjects. All patients and controls had a European Caucasian origin and were mainly from Northern Europe (91% MS, 88% controls).

PCR Amplification

DNA was amplified using primers, SP4.760 (CTTCATTACACCTGCAGCTCT) and PM6.942 (CACAGCCCTGTGCCTCTTCTTC) described by Liu et al. [10]. Genomic DNA (10–100 ng) was amplified in a 10 μl reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin (w/v), 1.5 mM $MgCl_2$, 250 μM dNTPs, 50 ng of each primer and 0.5 U AmpliTaq (Perkin Elmer). Amplifications were carried out in a capillary thermocycler (Corbett Research, Australia) using the following temperature profile: 1 cycle of 95°C for 3 min, 60°C for 15 s, 72°C for 15 s; 4 cycles of 94°C for 15 s, 60°C for 15 s, 72°C for 15 s and 35 cycles of 94°C for 3 s, 60°C for 3 s, 72°C for 15 s. PCR products were resolved on a 10% polyacrylamide gel. The 183 bp fragment of the normal CCR5 gene was easily resolved from the 151 bp fragment of the CCR5Δ32 gene. In addition, heterozygotes formed a distinctive heteroduplex pattern which was informative in the few instances when there were non-specific PCR products in the approximate size range of the 151 bp fragment.

Typing for Other Putative MS Susceptibility Genes

MS patients and controls were typed for HLA-DQA1 and -DQB1 alleles [22] by PCR-RFLP [23] and for TCRBV polymorphisms (Buhler et al. manuscript in preparation).

TABLE 1 CCR5 gene and genotype frequencies in MS patients and controls

				CCR5 Genotype Frequencies (%)				
		Gene Frequency *CCR5Δ32*	*p* nc	+/+	+/Δ32	Δ32/Δ32	*p* nc	RR Δ32 phenotype
All individuals								
MS	*n* = 120	0.1125	0.501	79	19	2	0.642	1.16
Controls	*n* = 168	0.0952		82	18	1		
*DRB1 * 1501 positive*								
MS	*n* = 73	0.1096	0.721	79	19	1	0.918	0.88
Controls	*n* = 44	0.1250		77	20	2		
*DRB1 * 1501 negative*								
MS	*n* = 47	0.1170	0.317	79	19	2	0.241	1.38
Controls	*n* = 122	0.0820		84	16	0		
DR1 positive								
MS	*n* = 17	0.0294	0.118	94	6	0	0.325	0.30
Controls	*n* = 36	0.1250		78	19	3		
DR7 positive								
MS	*n* = 22	0.1818	0.145	73	18	9	0.132	1.64
Controls	*n* = 43	0.0930		81	19	0		
*DQA1 * 0102 positive*								
MS	*n* = 61	0.1230	0.101	77	21	2	0.253	2.23
Controls	*n* = 51	0.0588		88	12	0		
*DQA1 * 0102 negative*								
MS	*n* = 38	0.1053	0.949	82	16	3	0.753	0.88
Controls	*n* = 88	0.1080		80	19	1		
*DQB1 * 0602 positive*								
MS	*n* = 52	0.1250	0.495	77	21	2	0.705	1.38
Controls	*n* = 28	0.0893		82	18	0		
*DQB1 * 0602 negative*								
MS	*n* = 44	0.1136	0.648	80	18	2	0.825	1.16
Controls	*n* = 99	0.0960		82	17	1		
*DQA1 * 0101 positive*								
MS	*n* = 15	0.0333	0.144	93	7	0	0.370	0.33
Controls	*n* = 39	0.1282		77	21	3		
*DQA1 * 0101 negative*								
MS	*n* = 84	0.1310	0.075	76	21	2	0.144	1.77
Controls	*n* = 100	0.0750		85	15	0		
DQA1 Glu 34 positive								
MS	*n* = 87	0.1034	0.204	80	18	1	0.343	1.56
Controls	*n* = 104	0.0673		87	13	0		
DQB1 Leu 26 positive								
MS	*n* = 86	0.1337	0.213	76	22	2	0.468	1.51
Controls	*n* = 102	0.0931		82	17	1		
*TCRBV3S1 * 1 positive*								
MS	*n* = 89	0.1000	0.798	80	20	0	0.787	1.11
Controls	*n* = 86	0.0930		81	19	0		
TCRBV3S1 * 2 positive								
MS	*n* = 92	0.1304	0.252	76	22	2	0.514	1.43
Controls	*n* = 111	0.0946		82	17	1		
*TCRBV6S5 * 1 positive*								
MS	*n* = 78	0.1474	0.037	73	24	3	0.097	2.12
Controls	*n* = 81	0.0741		85	15	0		
*TCRBV6S5 * 2 positive*								
MS	*n* = 100	0.0950	0.965	82	17	1	0.997	1.01
Controls	*n* = 112	0.0938		82	17	1		
*TCRBV8S1 * 1 positive*								
MS	*n* = 84	0.1190	0.965	79	19	2	0.482	1.49
Controls	*n* = 110	0.0818		85	15	1		
*TCRBV8S1 * 2 positive*								
MS	*n* = 92	0.0870	0.504	83	17	0	0.566	0.82
Controls	*n* = 93	0.1075		80	19	1		

TABLE 1 *Continued*

		Gene Frequency *CCR5Δ32*	*p* nc	CCR5 Genotype Frequencies (%) +/+	+/Δ32	Δ32/Δ32	*p* nc	RR Δ32 phenotype
*TCRBV10S1 * 1 positive*								
MS	$n = 98$	0.1122	0.553	80	18	2	0.758	1.17
Controls	$n = 111$	0.0946		82	17	1		
*TCRBV10S1 * 2 positive*								
MS	$n = 77$	0.0779	0.471	84	16	0	0.447	0.73
Controls	$n = 79$	0.1013		80	20	0		
*TCRBV15S1 * 1 positive*								
MS	$n = 93$	0.1022	0.466	82	16	2	0.463	1.20
Controls	$n = 70$	0.0786		84	16	0		
*TCRBV15S1 * 2 positive*								
MS	$n = 75$	0.0800	0.335	84	16	0	0.309	0.62
Controls	$n = 47$	0.1170		77	23	0		

+ = CCR5 wild type

Δ32 = CCR5Δ32 mutation

nc = not corrected

RR = Relative Risk

NB: DR typing available only for 167 controls. TCRBV3 typing available only for 131 controls. TCRBV6.5 typing available only for 118 MS patients and 131 controls. TCRBV8.1 typing available only for 132 controls. TCRBV10 typing available only for 117 MS patients and 129 controls. TCRBV15 typing available only for 113 MS patients and 80 controls. DQA1 typing available only for 99 MS patients and 139 controls. DQB1 typing available only for 96 MS patients and 127 controls.

HLA-DR typing was performed by a mixture of serological typing and genotyping (NSW Red Cross Tissue Typing Laboratories).

Statistical Analysis

Differences between the gene and genotype frequencies of CCR5 alleles in MS patients and controls were determined by χ^2 analysis. Relative risk was calculated by Woolf's method and by using Haldane's modification [24] where necessary to determine if the CCR5 phenotype added risk to DR, DQA1, DQB1 and TCRBV phenotypes.

RESULTS

The CCR5Δ32 gene frequency; genotype frequencies of wild type homozygotes, heterozygotes, CCR5Δ32 homozygotes; and relative risk of the CCR5Δ32 phenotype for all individuals, who were then stratified for a number of HLA-DR, -DQA1, -DQB1 and TCRBV phenotypes, are shown in Table 1. There was no significant difference in the allele frequency of CCR5Δ32 gene between the MS patients (0.1125) and the control population (0.0921). These allele frequencies are very similar to that observed in other Caucasian populations in France and Belgium (0.092, [11]) and in the USA (0.080, [9]; 0.098, [10]; 0.081, [12]). The CCR5 genotype frequencies showed no significant differences between the MS patients and the controls, and matched predicted Hardy-Weinberg equilibrium (MS observed 79%, 19%, 2%, expected 78%, 20%, 1%; Controls observed 82%, 18%, 1%, expected 82%, 17%, 1%).

CCR5Δ32/CCR5Δ32 Homozygotes with MS

The presence of two CCR5Δ32 homozygotes in the MS patient group indicates that the CCR5Δ32 gene is not protective against MS. These two patients were confirmed as having clinically definite MS and their clinical course was typical.

Correlation with MHC and TCRBV Phenotypes

A recent study in systemic lupus erythematosus has shown that there was an epistatic interaction between MHC class II and TCRB loci in determining high and low antibody responses to extractable nuclear antigen-derived peptides [25]. Given this and the known association between MS and MHC phenotypes [21] and putative associations between MS and TCRBV polymorphisms ([26–28], Buhler et al. manuscript in preparation), CCR5 gene and genotype frequencies were correlated with HLA-DR, -DQA1, -DQB1 and TCRBV phenotypes (Table 1). In addition, CCR5 gene and genotype frequencies were analysed in individuals possessing DQ alleles which have been postulated to share critical residues [29]. These were DQA1 alleles having a glutamine at residue 34 (DQA1 Gln34) and DQB1 alleles having a leucine at residue 26 (DQB1 Leu26). There were no significant associations found between

TABLE 2A Comparison of MHC and TCRBV phenotype frequencies (%) between CCR5Δ32 positive MS patients and CCR5Δ32 negative MS patients

Allele (HLA-DR)	All individuals (N = 120)	CCR5Δ32 positive (N = 25)	CCR5Δ32 negative (N = 95)	Allele (HLA-DQA1)	All individuals (N = 99)	CCR5Δ32 positive (N = 21)	CCR5Δ32 negative (N = 78)
DR 1	14	4	17	*0101	15	5	18
DRB1 * 1501	61	60	61	*0102	62	67	60
DR3	23	20	23	*0103	11	0	14
DR4	25	24	25	*0201	20	24	19
DR5	9	8	9	*0301	28	29	28
DR6	22	16	23	*0401	4	16	1
DR7	18	24	17	*0501	39	32	42
DR8	3	8	2	*0601	0	0	0
DR9	1	0	1				
DR10	1	0	1				

Allele (HLA-DQB1)	All individuals (N = 96)	CCR5Δ32 positive (N = 21)	CCR5Δ32 negative (N = 75)	Allele (TCRBV3S1)	All individuals (N = 120)	CCR5Δ32 positive (N = 25)	CCR5Δ32 negative (N = 95)
*0201	38	33	39	*1	74	72	75
*0301	23	14	25	*2	77	88	74
*0302	18	19	17	TCRBV6S5	N = 118	N = 25	N = 93
*0303	5	1	4	*1	66	84	61
*0304	0	0	0	*2	85	72	88
*0402	2	5	1	TCRBV8S1	N = 120	N = 25	N = 95
*0501	16	10	17	*1	70	72	69
*0502	1	0	1	2	77	64	80
*0503	1	0	1	TCRBV10S1	N = 117	N = 23	N = 94
*0504	0	0	0	*1	84	87	83
*0601	1	0	1	*2	66	52	69
*0602	54	57	53	TCRBV15S1	N = 113	N = 22	N = 91
*0603	17	10	19	*1	82	77	84
*0604/5	6	5	7	*2	66	55	69

CCR5 and any of the HLA-DR, -DQA1, -DQB1 and TCRBV phenotypes.

Further analysis was performed to determine whether the CCR5Δ32 polymorphism affected the relative risk of other known or suspected MS susceptibility genes. HLA-DR and HLA-DQ types and TCRBV polymorphisms were analysed in conjunction with the CCR5Δ32 polymorphism. There was no significant alteration to the relative risk conferred by these other putative MS susceptibility genes (Table 1).

Evolutionary Interaction

If the frequency of CCR5Δ32 is related to selective advantage involving immune function this may interact with specific MHC and/or TCRBV phenotypes. CCR5Δ32 positive and negative phenotypes were analysed in both MS patients (Table 2a) and controls (Table 2b) to determine if there was any distortion in HLA-DR, -DQA1, -DQB1 and TCRBV phenotype distribution. There was no significant alteration in the frequency of HLA-DR, DQA1, -DQB1 or TCRBV phenotypes with either the CCR5Δ32 positive or negative phenotype (p values not shown), indicating that there was no apparent selective pressure for the CCR5Δ32 polymorphism to go hand in hand with specific MHC or TCRBV phenotypes.

DISCUSSION

According to the arrangement of the first two cysteines, chemokines are divided into two sub families, the C-C chemokines and the C-X-C chemokines. The C-C chemokines such as MIP-1α, MIP-1β, RANTES and MCP-1, are chemotactic for monocytes, T-cells, basophils and eosinophils whilst C-X-C chemokines, such as MIP-2 are primarily chemotactic for neutrophils. Of the three ligands for CCR5, MIP-1α, MIP-1β and RANTES, the best evidence for chemokine involvement in MS and EAE to date relates to MIP-1α. High levels of MIP-1α were detected in the CSF of 8 of 13 patients with an acute relapse of MS compared to 1 of 5 patients in remission. MIP-1α was not found in the CSF of neurologic controls with non-inflammatory disease but

TABLE 2B Comparison of MHC and TCRBV phenotype frequencies (%) between CCR5Δ32 positive controls and CCR5Δ32 negative controls

Allele (*HLA-DR*)	All individuals (N = 167)	*CCR-5Δ32 positive* (N = 31)	*CCR-5Δ32 negative* (N = 136)	Allele (*HLA-DQA1*)	All individuals (N = 139)	*CCR-5Δ32 positive* (N = 24)	*CCR-5Δ32 negative* (N = 115)
DR 1	22	26	21	**0101*	28	38	26
*DRB1 * 1501*	26	32	25	**0102*	37	25	39
DR3	26	23	26	**0103*	11	13	10
DR4	25	23	26	**0201*	30	38	29
DR5	23	19	24	**0301*	29	21	30
DR6	26	19	28	**0401*	4	4	4
DR7	26	26	26	**0501*	43	42	43
DR8	4	6	4	**0601*	1	0	2
DR9	2	0	2				
DR10	1	6	0				

Allele (*HLA-DQB1*)	All individuals (N = 127)	*CCR-5Δ32 positive* (N = 23)	*CCR-5Δ32 negative* (N = 104)	Allele (*TCRBV3S1*)	All individuals (N = 131)	*CCR-5Δ32 positive* (N = 24)	*CCR-5Δ32 negative* (N = 107)
**0201*	41	39	41	**1*	66	71	65
**0301*	31	22	33	**2*	85	83	85
**0302*	12	4	13	*TCRBV6S5*	N = 131	N = 24	N = 107
**0303*	13	17	13	**1*	62	50	64
**0304*	1	0	1	**2*	85	83	86
**0402*	6	9	5	*TCRBV8S1*	N = 132	N = 25	N = 107
**0501*	23	30	21	**1*	83	68	87
**0502*	1	0	1	**2*	70	76	69
**0503*	7	9	7	*TCRBV10S1*	N = 129	N = 25	N = 104
**0504*	1	0	1	**1*	86	80	88
**0601*	1	4	0	**2*	61	64	61
**0602*	23	26	22	*TCRBV15S1*	N = 80	N = 14	N = 66
**0603*	6	9	5	**1*	88	79	89
**0604/5*	13	0	16	**2*	59	79	55

elevated levels were found in subjects with other inflammatory neurologic disorders. In addition, the level of MIP-1α in the CSF correlated strongly with the CSF leukocyte count [6]. In a study of EAE in SJL/J mice immunized with PLP, MIP-1α production correlated with the onset of clinical disease and to disease severity. This correlation was not seen with MCP-1 and MIP-2. Moreover, the administration of anti-MIP-1α on days 0 and 2 relative to the transfer of activated encephalitogenic T-cells prevented the development of the EAE. This was not seen with anti-MCP-1. The clinical benefit of anti-MIP-1α treatment was reflected in the histological evidence for little or no meningial, perivascular or parenchymal mononuclear cell infiltration of the spinal cord. Of clinical relevance, the administration of anti-MIP-1α to mice with the ongoing clinical EAE resulted in a significant decrease in the severity of clinical signs.

The importance of the entry of activated T cells into the CNS as a starting point for EAE has been known for some time following the demonstration that antibodies to the integrin VLA-4 inhibit binding of activated T-cells to vascular endothelium and, consequently, the development of the disease [30]. The role of chemokines in this regard may be in the up-regulation of VLA-4 and other adhesion molecules, although the full range of chemokine effects is yet to be defined.

The finding of two patients with MS who were homozygous for the *CCR5Δ32* deletion, and whose cells would not be expected to express CCR5 [10], indicates that this major C-C chemokine receptor is not essential in the pathogenesis of MS. Taken with the heterozygote data, which shows a slight increase in the frequency (not significant) of the *CCR5Δ32* mutation in the patient group, the CCR5 locus can be excluded as a significant candidate locus for susceptibility/resistance to the disease. This is further supported by the inability to find any significant correlation between CCR5 genotyping and MHC and TCR Vβ phenotypes. However, by analogy with HIV [11], it is possible that the heterozygous genotype may alter the rate of progression of an autoimmune disease like MS, although this question was unable to be addressed in this study.

If MIP-1α is important in the pathogenesis of MS, then the finding of individuals with this disease who

lack CCR5 (those homozygous for the *CCR5Δ32* mutation) indicates that it can exert its effect through binding to different chemokine receptors [16], possibly CCR1, CCR2b or CCR3. In addition, other chemokines utilising different receptors may influence what appears to be an integral step in the induction of EAE and probably MS: the homing of lymphocytes into the CNS. The results of our study indicate that further experiments using knock-out mice with respect to individual chemokine receptors may prove to be of considerable value.

If a specific infectious agent is essential in MS aetiology, then our data would indicate that CCR5 is either not the entry point for this infectious agent or its role can be replaced by other receptors in its absence. However, the role of chemokine receptor variants in allowing or not allowing infection of certain cell types raises the possibility that autoimmune susceptibility genes may function by either preventing or allowing infection in conjunction with other susceptibility genes such as the MHC.

In contrast to its high frequency in European Caucasian populations, the *CCR5Δ32* mutation has not been detected amongst Africans in Africa, Japanese, Asians and Venezuelans [10–12]. Its presence, at a very low frequency, in one study of African-Americans [9] is consistent with the known racial admixture. The high frequency of the *CCR5Δ32* mutation, which has arisen relatively late in human evolutionary terms, is not explained. It suggests the possibility of a survival advantage for individuals carrying the mutant gene, with respect to diseases which may have either been or are still prevalent within populations having a high frequency of this mutation. A similar situation has been described with the erythrocyte chemokine receptor (DARC), where a mutant allele which is not expressed in red blood cells provides resistance to *plasmodium vivax* [15]. There are a number of examples where there may be survival advantages for potentially deleterious mutations. For example, protection against tuberculosis has been postulated to be conferred on heterozygotes with a mutation in the hexoaminidase A gene while homozygotes develop Tay-Sachs disease [31]. Similarly, genes resulting in high levels of gastric secretion and predisposing individuals to ulcers may also protect against tuberculosis [32]; and genes responsible for non-insulin dependent diabetes mellitis may be beneficial when living on a subsistence diet but may induce diabetes when living on a food abundant, western diet [33]. If the *CCR5Δ32* mutation has influenced immune response to and, therefore, disease expression of an infectious agent, it is feasible to postulate that this may have selected individuals of a particular genotype of the MHC or TCR repertoire. The data gained from our study do not support this hypothesis. Protection against an autoimmune disease is a less likely explanation for the emergence and maintenance of a high level of mutation, unless that autoimmune disorder has an effect before the age of reproduction. If the current redundancy within the chemokine-chemokine receptor system has existed throughout evolution then perhaps a survival benefit from the absence of a single receptor, albeit a very important one, would be difficult to explain unless it is acting as an essential receptor for an infectious agent as is the case in HIV.

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A small molecular weight antagonist of CCR5 inhibits the development of CIA in rhesus monkeys.

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Short Title: A new CCR5 antagonist inhibiting rhesus monkey CIA

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Abbreviations used in this paper: CRP: C-reactive Protein; CIA: collagen-induced arthritis; CFA: complete Freunds'adjuvant; HP: hydroxylysylpyridinoline; s.c.: subcutaneous; LP: lysylpyridinoline; STS: soft tissue swelling.

Abstract

Collagen-induced arthritis (CIA) in the rhesus monkey is a widely investigated non-human primate model of rheumatoid arthritis (RA). The close phylogenetic relationship between man and rhesus monkey makes this model useful for the preclinical safety and efficacy testing of new therapies that are inactive in animals more distinctly related to humans. In the current study we have tested the therapeutic potential of a novel small molecular weight antagonist of CCR5 (SCH-X). Treatment with SCH-X or saline as control was initiated at the day of CIA induction resulting in reduced incidence and severity of CIA. Only two out of five animals in the treatment group displayed prominent soft-tissue swelling compared to five out of five animals in the group that received only saline. In addition to the suppression of joint inflammation, we observed a reduction of joint destruction by the treatment with SCH-X, as expressed by lower urinary excretion rates of collagen crosslinks and confirmed with histology. While in all saline-treated monkeys significant destruction of joint cartilage erosion was observed, this was absent in four out of five SCH-X treated monkeys.

Marked systemic effects of the treatment with SCH-X were a suppressed acute phase reaction (CRP) in the three treated monkeys that remained asymptomatic of CIA and an altered antibody response towards type II collagen. The results implicate that the CCR5 antagonist SCH-X might have a strong clinical potential for treatment during periods of active inflammation as seen in RA.

Introduction:

Chemokines are involved in the trafficking, localization and differentiation of leukocytes as well as in effector functions (for review see (1)). Besides the general feature of mediating chemotaxis of leukocytes to specific areas, some chemokines are only produced during conditions of inflammation and are held responsible for the attraction of cells towards and retention at inflammatory site(s). By the selective expression of chemokine receptors on specific (T) cell subsets, chemokines are believed to contribute to specific features of the inflammation process and the resulting tissue destruction by the attraction of cells with specific functional properties. This is illustrated by the preferential expression of CCR5 and CXCR3 on Th1-cells (2, 3) and of CCR4 and CCR3 on Th2 cells (4). Besides the selective expression on *in vitro* generated Th1 cell lines, target organs in Th1-associated autoimmune diseases, such as the central nervous system in Multiple Sclerosis (MS), the thyroid gland in Graves' disease and synovial joints in RA, were found enriched with T cells expressing CCR5 and/or CXCR3 (5-12). Moreover, increased levels of CCR5- and CXCR3-ligands have been detected in the areas of inflammation and/or the synovial fluid (SF) and cerebrospinal fluid (CSF) in respectively RA and MS patients (5, 7, 9, 10, 13, 14). Recently it was also functionally demonstrated that Th1 clones migrate more towards RA synovial tissue-derived supernatant than Th2 clones and that migration could be inhibited by reagents that block the action of CCL5 (15).

The presence of a non-functional CCR5 receptor, caused by a CCR5Δ32 mutant allele, is associated with a milder disease course in RA (16, 17) and with a later disease onset in the case of MS patients(18). This indicates that CCR5 and its ligands CCL5 (RANTES), CCL4 (MIP-1β), and CCL3 (MIP-1α) are potential targets for disease modifying therapy in chronic inflammatory diseases. Such strategies were found to be effective in rodent models of

collagen-type II arthritis (CIA) and adjuvant arthritis (AIA) either by using antibodies to chemokines (19, 20) or chemokine antagonists (21, 22).

In the current study we have tested a new small molecular weight antagonist specific for primate CCR5 (SCH-X) in a well-defined rhesus monkey model of CIA. The rationale for testing our compound in a non-human primate disease model is the exclusive reactivity with primate CCR5. Moreover, non-human primate models may better predict the clinical success of a new immunotherapy than the equivalent models in inbred strains of rats and mice (23). Our results show that the treatment of bovine type II collagen-immunized rhesus monkeys with the antagonist substantially reduces the severity and incidence of CIA in 4 out of 5 monkeys. The reduction of several disease markers, such as serum levels of C-reactive protein (CRP) and alkaline phosphatase and the urinary excretion rates of collagen crosslinks confirm the beneficial effect of the therapy. This study provides support for an efficacy evaluation of CCR5 antagonists in RA patients.

Materials and Methods:

Animals

Young adult, healthy male rhesus monkeys (*Macaca mulatta*), between 3 and 6 years of age and weighing 2.5 – 5.0 kg, were purchased from the Animal Science Department of the Biomedical Primate Centre (BPRC) in Rijswijk, The Netherlands. Animals susceptible to CIA were selected on basis of the absence of the dominant MHC class I resistance marker *Mamu-A26* (24).

During the study the animals were individually housed. They were fed with AM-II food-pellets (Hope Farms, Woerden, The Netherlands), rice, vegetables and fresh fruit of the season. Drinking water was provided *ad libitum* via an automatic watering system. Medication for pain treatment with Temgesic (Warrick, Amstelveen, The Netherlands; 0.1 to 0.3 ml per kg) was administered when indicated by the veterinarian staff. The ulcerative skin-lesions that develop at the immunization sites were treated with wound-spray (Acederm) each time an animal was sedated in order to prevent infection.

As specified in the Dutch Law on animal experimentation, the study-protocol has been reviewed and approved by the Institute's Animal Experimentation Committee. The study protocol was also approved by the SPRI Animal Care and Use Committee.

CIA induction

To induce CIA, monkeys were inoculated into the dorsal skin with 5 mg of bovine collagen type II that was extracted from hyaline cartilage as an emulsion in complete Freund's Adjuvant (CFA; DIFCO Laboratories, Detroit, MI)(25). In brief, lyophilized collagen was dissolved in 0.1 M acetic acid to a concentration of 10 mg/ml and emulsified in an equal volume of CFA (CII/CFA) to a final concentration of 5 mg/ml. Each monkey was injected

into the dorsal skin with 1 ml of the emulsion, distributed over 10 spots (100 µl per spot) to reduce the ulcerative skin lesions.

Characterisation of the CCR5 antagonist SCH-X

SCH-X was obtained from the Department of Chemistry, SPRI (Baroudy B. et al. 2002. United States Patent # 6,391,865 B1). Mouse Ba/F3 cells stably transfected with an SRα-promoter-based plasmid (26) containing rhesus monkey CCR5 (Genbank Accession No. AF124381) were established by geneticin (1 mg/ml) selection and used for characterizing the antagonist activity of SCH-X. Human MIP-1β (R&D Systems, Minneapolis, MN, USA) was reconstituted in Dulbecco's phosphate-buffered saline (PBS) as a 20 µM stock. For titration of hMIP-1β in chemotaxis of the cells, the chemokine was used in serial dilutions. For antagonist assay, hMIP-1β was used at a constant final concentration of 0.5 nM. Chemotaxis assay was carried out by modification of a protocol previously described (27). The buffer for both chemokine dilution and cell resuspension consisted of Ba/F3 culture medium (26) diluted at 1:10 ratio into RPMI1640. The assay was performed according to the manufacturer's instructions using 96-well ChemoTx microplates (NeuroProbe, Inc, Gaithersburg, MD, USA) with a 5 µM filter. The tests of chemokines or compounds were carried out in duplicates. The test compound was present in both the bottom and the top wells; chemokine was present in the lower wells only. Cells were dispensed in the upper wells at 6.25 x 10^4 cells per 25 µl per well. The microplates were placed in a 37°C humidified CO_2 (5%) incubator for 2 hours to allow cells to migrate toward the chemokine. Cell migration was quantitated by CellTiter-Glo Luminiscent Cell Viability Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. The IC50 values were calculated by using EXCEL and GRAPHPADPRISM software.

Study design

First we determined plasma levels and clearance profiles of the antagonist in two monkeys (Ri207 and Ri210). These were injected with a single subcutaneous dose (3 mg/kg) of SCH-X under light ketamin sedation (0.1 ml/kg intramuscular). Just prior to dosing, and subsequently at 0.5- 1- 2- 4- 6- 8- 12- 24- 48- 72 hours post-dosing 1 ml blood samples were collected from the femoral vein into vacutainers with EDTA as anti-coagulant (Becton Dickinson). After centrifugation for 10 minutes at 600 g the blood plasmas were collected and stored frozen at -80 ^{0}C until analysis. Plasma levels of SCH-X were determined at the department of Bioanalysis at TNO-Nutrition (Zeist, The Netherlands). In brief, plasmas were analyzed with HPLC using a Waters Symmetry C18 column (50 x 3.9 mm, 5 μ particles, injection volume 75 μl) with an APCI mass spectrometer as detector (detection limit 10 ng.ml). 10 mM NH_4Ac in 52% methanol was used as eluent at a flow rate of 0.6 ml/min. After washing with 0.6 ml of the above buffer, the compound was eluted from the column with 1.8 ml 10 mM NH_4Ac in 92% methanol.

Next we determined the anti-arthritic effect of SCH-X in the rhesus monkey CIA model. CIA was induced in 10 monkeys, which were randomly allocated to the two treatment groups consisting each of 5 monkeys. Note that monkey Ri207 had been used in the pharmocokinetic study and was therefore assigned to the placebo group. SCH-X (2 mg/kg; twice daily, one injection in the early morning, one in late afternoon) or an equivalent volume of the solvent were injected subcutaneously (s.c.) into the flanks. The dosing was initiated immediately after the induction of CIA and continued for 45 days. Monkeys were monitored until 63 days after CIA induction, to assess a possible delayed onset of CIA, unless humane killing was needed because of the severity of their disease. Three monkeys had to be euthanized before the end-point of the study with severe CIA, namely two sham-treated monkeys (Ri154, R604) at 29 days and one SCH-X-treated monkey (Ri173) at 45 days after immunization.

Clinical assessment:

Macroscopic signs of clinical arthritis, soft tissue swelling (STS) and redness of affected joints, were scored twice weekly using a previously published semi-quantitative scale (28): 0 = no disease symptoms; 0,5 = fever; 1 = apathy and loss of appetite, weight loss; 2 = warm and tender joints, but without soft tissue swelling; 3 = moderate STS but normal flexibility of affected joints; 4 = severe STS with joint stiffness; 5 = such severe disease that euthanasia is necessary. Each time the monkeys were sedated, they were weighed and their body temperatures were recorded. Simultaneously the number of joints with visible soft-tissue swelling (STS) were scored.

Urinary excretion rates of collagen cross-links

Urinary excretion rates of the collagen crosslinks hydroxylysylpyrridinoline (HP) and lysylpyrridinoline (LP) were determined twice weekly, starting from the day of CIA induction. For that purpose each animal's urine was collected overnight in a fine-maze covered tray placed under the cage. After precipitation of debris by centrifugation the clear supernatant was isolated and stored frozen at - 20°C until analysis. Reverse-phase HPLC was used to determine HP and LP levels in hydrolyzed urine samples as described (29, 30). The levels of HP and LP were normalized to creatinine levels (nmol levels per mmol creatinine) to compensate for a possible dilution-factor due to drinking water spilled into the urine. To account for different baseline excretion rates between individual monkeys, HP and LP levels are expressed relative to the value at the start of the study which was set at 1. In case of LP levels of monkey Ri173, these were normalized to day 10 as abnormal values were found on previous days.

Serum levels of bovine collagen type II antibodies:

Serum concentrations of anti-bovine CII IgM and IgG antibody were detected by ELISA as described (25). In brief, flexible 96 well plates (Falcon 3911, Becton and Dickinson, San Jose, CA) were coated overnight at 4°C with 100 µl/well of 1 mg/ml bovine CII in PBS. Plates were washed 5 times with PBS + 0.05 Tween-20 and blocked with 200 µl/well of PBS + 2% bovine serum albumin (BSA) at 37°C, 5 % CO_2 in a humidified atmosphere for 2h. Subsequently, wells were filled with 100 µl of diluted serum (1:200 for IgM; 1: 1600 for IgG) in PBS + 1% BSA and incubated overnight at 4°C. After extensive washing, the wells were incubated for 2 h at 37°C with 100 µl alkaline-phosphatase conjugated goat anti-hIgM 1 : 10.000 (or goat anti-hIgG 1 : 1.000) in PBS + 1% BSA or (Biosource, Camarillo, CA). After washing, Ab-binding was detected with the addition of 100 µl/well of poly-nitrophenylphosphate (pNPP) diluted in Tris buffer (Sigma Chemicals). After 30 minutes the reaction was stopped by addition of 100 µl of 0.1 M H_2SO_4 to each well and the color determined at 405 nm.

Haematology and serum chemistry

Blood samples for serum chemistry and haematology were collected once weekly under ketamin sedation. All analyses were performed at The Laboratory Centre for Clinical Chemistry SSDZ (Delft, The Netherlands).

Histology

At necropsy, the fingers and toes were removed and fixed in 4% phosphate-buffered formalin. After fixation, the bones were decalcified for at least 3 weeks in Kristensen's solution (17% formic acid in 1 M NaOH, pH 2.2). Subsequently, bones were washed in tap water for 16 h and dehydrated in ethanol/toluene and embedded in paraffin. Sections of 2 µm thick were cut

and stained with HPS (haematoxylin-phloxin-saffran) or Alcian Blue-PAS (periodic acid Schiff). Of arthritic monkeys all affected interphalangeal joints were examined. Of the non-arthritic animals the third digits of hands and feet were removed and fixed in 4% phosphate-buffered formalin.

Statistics:

Statistical differences were determined by using the Mann-Whitney U test.

Results

Inhibition of the monkey CCR5-mediated cell migration by SCH-X

We first assessed the activity of SCH-X (Figure 1A) in inhibiting the biological function of the rhesus monkey CCR5. Due to the unavailability of monkey CCR5 ligands, human MIP-1β was used as a surrogate chemoattractant. The recombinant cells stably expressing monkey CCR5 responded to and migrated toward the MIP-1β with an EC_{50} of about 0.3 nM (Figure 1B). The cells were then tested in the presence of SCH-X, with a constant concentration of the chemoattractant (0.5 nM). Under the test conditions we found that SCH-X inhibited MIP-1β induced chemotaxis with an IC_{50} of 3.9 nM (Figure 1C). Therefore SCH-X was determined to be a potent antagonist for the monkey CCR5.

Plasma levels of the CCR5 antagonist

Two monkeys (Ri207 and Ri210) received a single dosage of 3 mg/kg of SCH-X and the plasma levels were determined at different time-points. Peak circulating levels were detectable within 1h after injection and declined to background values within 48 h (Fig.2A). On the basis of the wash out profile, the plasma half-life of SCH-X was estimated at about 5 h. On the basis of the pharmacokinetic profile, we decided to dose the monkeys in the efficacy study twice daily (6-8h apart) with 2 mg/kg of the CCR5 antagonist. This dosing regimen was chosen to ensure adequate receptor coverage based on the 5 nM (3 ng/ml) Ki of SCH-X for rhesus monkey CCR5 (data not shown). Circulating levels throughout the efficacy study were determined in blood samples that were collected once weekly. Serum levels between 20 - 40 ng/ml were measured in all monkeys throughout the treatment period (Fig.2B) and no major differences were found between individual animals. Notably, drug levels of 40 ng/ml predict approximately 93% receptor occupancy. After cessation of the dosing, serum levels rapidly declined under the detection-limit (data not shown).

Effect of SCH-X on clinical and histological features of CIA

CIA in rhesus monkeys presents clinically as a monophasic episode with moderate to severe and often symmetrical inflammation of the wrists/ankles/knees and the small joints (IP) of the hands and feet. The disease usually starts within 3-5 weeks after disease induction with increased serum CRP levels and weight-loss and may last for several weeks(30). We have started treatment with SCH-X at the day of CIA induction and continued dosing until day 45 when all monkeys in the control group (5 animals) still showed clinical signs of CIA, namely STS and/or weight-loss.

Placebo group: 4 out of 5 monkeys (Ri162 excepted) lost more than 5% of their body weight (Fig. 3C), and 5 out of 5 displayed a gross arthritis score of 3 or higher (Fig.3A). As for the clinical course, two monkeys of this group developed typical acute onset CIA with a high number of inflamed joints (28 in Ri173 and 27 in R604; Fig.3E) and a sharp decline of their bodyweight. For ethical reasons these two animals were sacrificed on days 29 (R604) and 45 (Ri173), respectively. Histological examination confirmed the severe arthritis. Fig.4F shows the hyperplastic synovium, the pannus tissue that overgrows the joint cartilage and the extensive deformation of cartilage and subchondral bone in the severely arthritic monkey Ri173. CIA in the three other monkeys was characterized by a more gradual decline of bodyweight (Figure 3C) and less affected joints (Ri207:10; Ri162: 11; Ri171: 9; Fig. 3E). The histological characterization of the disease in these three monkeys was as follows: Ri171 (Fig.4G), chronic arthritis with lymphocyte and neutrophil infiltrates, synovial hyperplasia and considerable damage of the cartilage; Ri162 (Fig.4H), chronic arthritis with lymphocyte infiltrates and focal/segmental loss of cartilage; and Ri207 (Fig.4I), mild arthritis with lymphocyte and neutrophil infiltrates and moderate synovial hyperplasia in several joints.

SCH-X treatment group: In 2 out of 5 monkeys (Ri154 and Ri158; Figure 3B) clinical CIA developed before day 35, while 3 monkeys never developed any overt clinical signs of CIA (clinical score >2) during the whole observation period of 63 days. CIA in monkey Ri154 was characterized by an acute onset, a sharp decline of the bodyweight (Figure 3D) and a high number of affected joints (Figure3F; 31). This monkey was sacrificed at day 29. Histological examination revealed strong synovial hyperplasia with prominent deformation of cartilage and subchondral bone (Fig. 4A). The second arthritic monkey (Ri158) displayed less severe clinical signs, namely only a minor decline of bodyweight and a low number (maximum of 6) inflamed joints. Histologically this monkey displayed in one interphalangeal joint moderate chronic arthritis with segmental loss of cartilage, without cellular infiltrates (Figure 4B). Of the 3 asymptomatic cases, monkeys Ri155 and Ri156 displayed weight-loss (Figure 3D) at the time-point that CIA developed in the placebo group, while no weight-loss was observed in monkey RI151. The mean clinical CIA score and the mean number of joint with STS were significantly lower in the SCH-X group as compared placebo group ($P = 0.027$, d0-d63 for CIA score; $P = 0.045$, d0-d63 for number of joint with STS). Histological examination of the interphalangeal joints and patellae demonstrated either no alterations (Ri151 and Ri156; Figure 4C-D) or the presence of only few single lymphocytes in the synovialis and patellae in the absence of any visible cartilage destruction (Ri155; Figure 4E).

Effect of SCH-X on markers of CIA

Several biomarkers have been used to confirm the beneficial effect of SCH-X on the rhesus monkey CIA model. Plasma levels of CRP and urinary excretion rates of the collagen crosslinks HP and LP serve as surrogate markers of the inflammation and tissue destruction in the rhesus monkey CIA model and were found particularly useful in preclinical trials of new therapies (30, 31). Episodes of clinical activity in CIA are also marked by an increased serum

concentration of alkaline phosphatase (AP), possibly due to increased bone metabolic activity (32). Moreover, a consistent systemic reaction observed in CIA affected monkeys involve increased numbers of neutrophilic granulocytes and platelets in the circulation.

CRP (Fig. 6A/B): As anticipated, the placebo-treated monkeys with acute CIA had the highest CRP concentration in their serum (Figure 6A), while levels were lower in those with less acute disease. The CRP serum levels of the two SCH-X-treated monkeys, which developed arthritis (Figure 6B; Ri154 and Ri158) were in the same range as those, measured in the control animals. In the three SCH-X-treated monkeys, which remained asymptomatic, the normally observed increase of serum CRP-levels did not take place. The cumulative CRP-levels measured during the episode of clinically active CIA (d14-d50) were significantly lower in the SCH-X group than in the Placebo group.

Neutrophils (Fig. 6E/F): It is shown that in all placebo-treated monkeys the neutrophil counts increased 2- to 2.5-fold (Figure 6E). Although slightly higher neutrophil numbers were counted in the SCH-X-treated group (Figure 6F; up to 4-fold increment), this difference was not statistically significant relative to the placebo-treated group.

Alkaline phosphatase (Fig. 6C/D): In the two placebo-treated monkeys with acute CIA (Figure 6C; Ri604 and Ri173) as well as in one placebo-treated monkey with less acute CIA (Ri162) substantially increased serum AP levels were measured. Much lower changes were found in the two other monkeys with less acute CIA. In the SCH-X-treated group (Figure 6D) increased serum concentrations of AP were detected in the two monkeys that developed arthritis. However, sera from the three monkeys that remained asymptomatic did not contain elevated levels of this marker.

Urinary excretion rates of collagen crosslinks:. Markedly increased urinary excretion rates of HP (Figure 5A; 9-14 fold) and LP (Figure 5C; 4 -8 fold) were found in two placebo-treated monkeys with acute CIA. In 2 of the 3 monkeys with less acute CIA a lower, but still

detectable increase of the HP excretion rate (3-4 fold) was found (Fig.5A). In the group of monkeys that was treated with SCH-X we measured elevated urinary excretion of HP only in the one monkey that developed severe clinical CIA (Figure 5B), although the level was much lower than in the two placebo-treated monkeys with acute CIA.

Rhesus monkey anti-bovine CII antibody response

The susceptibility of rhesus monkeys to CIA is related to the capacity to produce adequate amounts of IgM antibodies to the challenging antigen (25). Experimental down-regulation of IgM autoantibody production leads to CIA resistance (33). For this reason it is relevant to measure the possible effect of SCH-X on the production of autoantibodies.

We found no significant difference in the absolute serum levels of anti-bovine CII IgM antibodies between monkeys in the control group (Figure 7A) and monkeys treated with the CCR5 antagonist (Figure 7C). However, in three of the SCH-X treated monkeys (Ri151, Ri156, and Ri158) the anti-bovine CII IgG levels remained low (Figure 7D). Only in monkey Ri155 we found a normal IgG profile, while monkey Ri154 was sacrificed before a detectable IgG response is normally observed in this model. Despite this observation, the presence of a lower anti-bovine CII IgG Ab response did not correlate with the absence of clinical signs as in monkey Ri155. This monkey displayed a normal IgG response, but remained free of clinical signs, whereas monkeys Ri158 developed CIA although it contained a lower serum level of anti-bovine CII IgG antibodies.

Discussion

The pathogenesis of rheumatoid arthritis (RA) is driven by Th1-cells, which are activated in peripheral lymphoid organs and subsequently recruited into the joints (34, 35). Most of the current therapies for RA suppress immunological functions in a rather nonspecific manner. A more attractive approach would be the selective prevention of joint infiltration by immune cells, thereby leaving the capacity to combat infections intact. Leukocytes are attracted to sites of inflammation by the interaction of locally produced chemokines with receptors on the migrating cells (36). To date, more than 40 chemokines have been described, which interact with a total of 18 receptors (37). Of particular interest for RA is the CC chemokine receptor 5. Known ligands of CCR5 include CCL5 (RANTES), CCL4 (MIP-1β), and CCL3 (MIP-1α), which have proven chemotactic activity in arthritis models. Treatment with a synthetic CCR5 antagonist was found to protect DBA/1 mice from CIA (22). Finally, increased numbers of CCR5+ T-cells were found in the synovium/synovial fluid in RA (12, 38). CCR5 appears to be preferentially expressed on Th1 cells, which in animal models of RA encompass the arthritogenic T-cell specificities.

This publication describes the beneficial effect of a novel antagonist of human CCR5, named SCH-X, in the rhesus monkey model of CIA. The antagonist fails to bind mouse or rat CCR5, but cross-reacts significantly with cloned CCR5 of non-human primate species, namely common marmosets (*Callithrix jacchus*) and rhesus macaques (*Macaca mulatta*). As no validated arthritis model exists in common marmosets we have tested the efficacy of SCH-X in the rhesus monkey CIA model. CIA in rhesus monkeys has proven to be a useful experimental model of RA for the preclinical efficacy evaluation of new anti-arthritic therapies (39, 40).

As the rhesus monkeys used in this experiment are derived from an outbred population, a similar variation of disease susceptibility and clinical course is observed

between individual monkeys as among RA patients (24, 41). Nevertheless, the pathogenic mechanisms that operate in the rhesus monkey CIA model seem to a large extent comparable to more broadly used CIA models in inbred strains of rats and mice. Inhibition of T-cell activation with cyclosporin A (42) or a humanized antagonist antibody to the human IL-2 receptor (31) as well as modulation of the autoantibody response (33) protects monkeys from the clinical and pathological consequences of CIA. This suggests that the inflammation and erosion of synovial joints is caused by a similar synergistic action of anti-CII T-cells and autoantibodies as has been described in the equivalent rodent CIA models (43-45).

The infiltration of knee joint synovium of CIA-affected monkeys with T-cells and macrophages seems to occur well before outward signs of joint inflammation are visible (46). For this reason we have chosen to start treatment with the antagonist already at an early stage in the CIA pathogenesis. Early treatment with the CCR5 antagonist, however, might affect the normal development of a pathogenic autoimmune reaction. The capacity to produce adequate amounts of IgM autoantibodies in particular was identified as a critical factor in the induction of clinical arthritis in rhesus monkeys as well as in rodent models of CIA (25, 47). However, our results showed no major differences between anti-CII IgM antibody responses in treated and control monkeys. A difference was found at the level of anti-CII IgG antibodies, but this did not correlate with the expression of clinical signs.

The chosen treatment regimen with SCH-X was well tolerated by the rhesus monkeys as it caused no overt adverse side effects, neither on the general well-being nor on hematological parameters (data not shown), and had a clear beneficial effect on the arthritis. Four out of five monkeys receiving two injections per day with the CCR5 antagonist from the day of CIA induction displayed clearly less severe arthritis than sham-treated monkeys, either expressed as gross clinical score or as the number of arthritic joints. The clinical observations were confirmed by positive effects on more objective disease markers of inflammation, such

as bodyweight, serum CRP concentration, although blood neutrophil counts were unaffected. The suppressive effect of SCH-X on the acute phase reaction has not been further investigated, but may be mediated via IL-6 that is produced by macrophages and by synovial fibroblasts upon ligand binding to the CCR5 receptor (48-52).

With regard to the joint histology we observed in all placebo-treated monkeys clear synovitis and, possibly with the exception of Ri207, marked destruction of cartilage and bone. However, the increased serum concentration of alkaline phosphatase and the increased urinary excretion rate of the collagen cross-links HP suggest that (moderate) joint pathology is present in monkey Ri207. With the exception of Ri154 the joint histology of the SCH-X-treated monkeys showed mild or no signs of arthritis. In three of the four asymptomatic monkeys (monkey Ri208 excluded) this was confirmed by the observation that serum alkaline phosphatase concentration and urinary excretion rates of HP, were consistently elevated.

In conclusion, the results of the current study show the anti-arthritic potential of a new CCR5 antagonist in an outbred cohort of CIA-affected rhesus monkeys. The antagonist seems to act on both arms of the disease, i.e. joint inflammation and erosion, most likely by preventing the joint infiltration by pathogenic immune cells. Taken together, these results provide a rationale for the clinical evaluation of CCR5 targeted therapeutics in RA.

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Legends:

Figure 1: *Structure and in vitro antagonist activity of SCH-X.*

A). The structure SCH-X. **B).** Chemotaxis of Ba/F3-monkey CCR5 cells toward hMIP-1β. C). Inhibition of hMIP-1β (0.5 nM)-induced chemotaxis of Ba/F3-monkey CCR5 cells by SCH-X.

Figure 2: *Plasma levels of SCH-X*

A) Two animals (○ Ri207;● Ri210) received a single subcutaneous injection of 3 mg/kg SCH-X dissolved in PBS. Plasma levels (ng/ml) were determined at the indicated time-points as described in the materials and methods section. B) Immediately after induction of CIA five preselected monkeys received s.c. dosages of 2 mg/kg SCH-X twice daily (6-8 h apart). The dosing was continued until day 45. Blood samples were collected at 7 days interval and plasma levels (ng/ml) were determined as described in the materials and methods section. The measured levels at day 0, day 45 and day 49 were below the detection-limit of the assay ($\leq$10 ng/ml) and are therefore not depicted. Symbols: ● Ri151; ◆ Ri155, ▲ Ri156, ■ Ri158, ⬟ Ri 154.

Figure 3: *Effect of prophylactic treatment with SCH-X on the clinical signs of CIA.*

Of a total of ten monkeys in which CIA was induced, five preselected monkeys received twice daily s.c. injection of 2 mg/kg SCH-X twice daily (6-8 h apart) and five others received s.c. injection of saline at the same volume-to-bodyweight ratio. Dosing was started at the day of CIA induction and continued until day 45. Unless a monkey was euthanized for ethical reasons, it was observed until day 63 in order to assess a possible delayed arthritis onset. Macroscopic signs of active arthritis, such as soft-tissue swelling (STS) and redness of affected joints were scored daily using a semi-quantitative scale (**A/B**) as follows: 0 = no

disease symptoms; 0,5 + fever; 1 = apathy and loss of appetite, weight loss; 2 = warm and tender joints, but without STS; 3 = moderate STS but normal flexibility of affected joints; 4 = severe STS with joint stiffness; 5 = such severe disease that euthanasia is necessary. Two times per week the monkeys were weighed, the bodyweight serves as a surrogate disease marker (**C/D**), and simultaneously the number of joints with visible STS (**E/F**) were scored. Bodyweight in time is depicted as the percentage of weight-loss from day 0 (**C/D**). The cumulative clinical CIA score and numbers of joints with visible STS was significantly lower in the SCH-X treated group (B and F) as compared to Placebo treated animals (A and E) from day 10-63 ($P<0,028$ clinical CIA score, $P<0,015$ number of joints with STS). The loss of body weight during day 7-63 was not statistical significant different between the SCH-X treated group (D) and the Placebo treated animals (C).

Symbols SCH-X treated animals : ● Ri151; ◆ Ri155; ▲ Ri156; ■ Ri158; ✚ Ri 154.

Symbols Placebo treated animals: ○ Ri162; ◇ Ri171; △ Ri207; □ Ri173; ✪ Ri 604.

Figure 4: *Effect of prophylactic treatment with SCH-X on the histopathology of CIA.*

Pathomorphological changes in CIA-affected proximal interphalangeal (PIP) joints of monkeys treated with saline (Ri162, Ri171, Ri207, Ri173, Ri604) or SCH-X (Ri151, Ri155, Ri156, Ri158, Ri154) were visualized by PAS staining. All magnifications are 100 x;.

Placebo group; PIP joint of monkey **Ri173** displaying extensively proliferated synovium, overgrowing the jojnt cartilage; PIP joint of monkey **Ri171** showing activated bone marrow, alignment of osteoclasts and osteoblasts, proliferated synovium and complete erosion of joint cartilage; In monkey **Ri162** the PIP joint displayed cartilage erosion and active synovium; Mild pathology of the PIP joint of monkey **Ri207** with activated synovium but without cartilage erosion. The PIP joint pathology in monkey R604 was comparable with that of monkey Ri154 (data not shown).

SCH-X group: Monkey **Ri154** shows severely eroded cartilage and signs of bone-remodeling with hyperplastic synovium that is infiltrated with mononuclear cells; Monkey **Ri158** displays minor pathology with some segmental cartilage loss and some synovial activation; Monkey **Ri151** displays healthy cartilage and inactive synovium; PIP joint of monkey **Ri155** without any abnormalities; PIP joint of monkey **Ri156** showing a healthy cartilage and synovium with some inflammatory cells.

Figure 5: *Effect of prophylactic treatment with SCH-X on urinary excretion rates of collagen crosslinks.*

Urine samples were collected twice weekly and the excretion rates of the major collagen-crosslinks HP (**A-B**) and LP (**C-D**) were determined as described in the materials and methods section. Urinary excretion rates were normalized to creatinine levels to compensate for a possible dilution factor and expressed as relative to the value at the start of the study (value at day 0 = 1). In case of LP levels of monkey Ri173, levels were expressed as relative to day 10 as abnormal values were found at earlier days. The cumulative relative HP excretion-rate of the SCH-X treated group (**B**) from day 10 until day 56 was significant lower than in the Placebo treated group (**A**; P<0,016). Cumulative relative LP excretion-rate levels were not significantly different between the SCH-X treated group (**C**) and the Placebo treated group (**D**). Symbols SCH-X treated animals : ● Ri151; ◆ Ri155; ▲ Ri156; ■ Ri158; ✚ Ri 154.

Symbols Placebo treated animals: ○ Ri162; ◇ Ri171; △ Ri207; □ Ri173; ⬡ Ri 604.

Figure 6: *Effect of prophylatic treatment with SCH-X on biomarkers of CIA*

Episodes of clinically active CIA are associated with changes of blood parameters. Of these, absolute numbers of platelets and neutrophils and the serum concentration of C-reactive protein (CRP) serve as biomarker of inflammation. The increased metabolic activity of bone

during periods of bone destruction causes increased serum concentration of alkaline phophatase (AP). Serum levels of CRP were determined twice weekly (every 3-4 days) and AP and absolute numbers of neutrophils and platelets in the peripheral blood were determined once weekly as described in the materials and methods section. There was a tendency for lower cumulative CRP-levels in the SCH-X treated group (**B**) during the periods from day 21 until day 45 and from day 49 until day 63 as compared to the Placebo (**A**) treated group ($P<0,08$; analysis with exclusion of monkeys R604 and Ri154 as they were euthanized earlier). The increment in serum AP levels (**C** and **D**) and in absolute numbers of neutrophils (**E** and **F**) or platelets (**G** and **H**) in the peripheral blood are depicted as relative to the value at day 0. In all the monkeys in the Placebo treated group an increment in serum AP was detected during the period of clinically active disease whereas in the SCH-X treated group only the 2 of the 5 monkeys with clinical evident CIA (Ri154 and Ri158) displayed a clear increment. The increment in absolute neutrophil and platelet numbers during the onset of CIA (day 7-21) was observed in all monkeys in the Placebo treated group and in 4 of the 5 monkeys in the SCH-X treated group. During this period, the increment in neutrophils tended to be higher in the SCH-X treated group although not significantly. Symbols SCH-X treated animals :

● Ri151; ◆ Ri155; ▲ Ri156; ■ Ri158; ✤ Ri 154. Symbols Placebo treated animals:

○ Ri162; ◇ Ri171; △ Ri207; □ Ri173; ⌬ Ri 604.

Figure 7: *Effect of prophylactic treatment with SCH-X on absolute serum levels of anti-collagen type II IgM and IgG antibodies.*

Anti-bovine CII IgM and IgG antibody levels were determined one weekly as described in the materials and methods section by means of ELISA. Absolute serum levels of the IgM antibody response in the Placebo (**A**) and SCH-X treated group (**B**) were not different. The

kinetics of the IgM response are similar in both groups. In 4 monkeys out of the Placebo treated group (**C**) absolute serum levels of IgG antibodies above 0,6 were detected. Absolute serum levels of IgG antibodies remained below a titter of 0,6 in the 2 out of 3 monkeys in the SCH-X treated group that did not develop clinical evident CIA (Ri156 and Ri158). Monkeys Ri154 and R604 were euthanized before high IgG antibody levels could have been detectable.

Symbols SCH-X treated animals: ● Ri151; ◆ Ri155; ▲ Ri156; ■ Ri158; ✿ Ri 154.

Symbols Placebo treated animals: ○ Ri162; ◇ Ri171; △ Ri207; □ Ri173; ✿ Ri 604.

Table 1: *Individual data of the monkeys*

Monkey code	Sex	Date of birth (dd-mm-yy)	Bodyweight (g)[@]
Pharmacokinetic experiment			
Ri207	M	11-11-96	3300
Ri210	M	03-07-96	3700
Control group			
Ri162	M	05-05-95	6150
Ri171	M	28-09-96	4100
Ri173	M	11-05-96	4750
Ri207	M	11-11-96	3300
R604	M	03-06-94	7850
SCH-X treatment group			
Ri151	M	26-10-96	3550
Ri154	M	10-05-96	4500
Ri155	M	24-07-96	6350
Ri156	M	28-03-96	3950
Ri158	M	27-07-96	4850